MODULATION OF NOTCH SIGNALING COMPONENTS IN PRESENCE OF EPIGENETIC MODULATORS IN BREAST CANCER

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CERTIFICATE

This is to certify that the thesis entitled "**MODULATION OF NOTCH SIGNALING COMPONENTS IN PRESENCE OF EPIGENETIC MODULATORS IN BREAST CANCER**" which is being submitted by **Mr. Tapas Tripathy**, Roll No. 411LS2126, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by him under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I hereby declare that this project report on, **"MODULATION OF NOTCH SIGNALING COMPONENTS IN PRESENCE OF EPIGENETIC MODULATORS IN BREAST CANCER"**, is the result of the work carried out by me .Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. The work was done under the guidance of ***Dr. Samir Kumar Patra , Associate Professor and Head, Department of Life Science, National Institute of Technology, Rourkela.***

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ABSTRACT

Notch signaling is one of the pleotropic signaling pathway that plays key role in development as it promotes differentiation and tissue morphogenesis. In recent years it has been observed that this pathway along with many other developmental pathways is seen to be deregulated in different cancers. The role that is played by this pathway is highly context dependent in cancers that is in some cases it act as a tumor suppressor while as oncogenic in others. It is proved with its oncogenic role in all forms of breast cancer. We tried to assess the effects on this pathway in MDA MB-231 breast cancer cells after treatment with epigenetic modulators S-Adenosyl Methionine (SAM) and S-Adenosyl Homocysteine (SAH) in a time dependent manner for 0-48 hr. The expression of important notch receptor Notch-1 and downstream effector Hes-1 were shown to be down regulated after SAM treatment but the SAH treatment upregulated them. This was accompanied by apoptotic induction in SAM treated cells in a more aggravated manner as compared to the SAH treated cells. From all these results we tentatively conclude that SAM treatment of invasive breast cancer cell line induces cell death in a notch dependent manner.

INTRODUCTION

The lifetime risk of developing breast cancer is about one in nine for women with around 1,41,000 new cases being diagnosed in the world wide each year. This has been proved recently as the worst and fatal breed of cancers in females. However, a significant decrease in the mortality since 1989 due to rigorous research, screening, specialization of care, and the widespread adoption of *tamoxifen* along with other effective anti-cancer therapeutics. More recently, number of Epigenetic therapeutics have emerged as new hopes for successful therapy of many cancers along with breast cancer working by altering the oncogenic epigenetic modifications that are one of the root causes of cancers. Despite this, there are still just short of 13,000 deaths each year due to breast cancer in western countries, suggesting that a much more in-depth understanding of the disease is required to improve treatment. Here in this study we have tried to test the anti-cancer efficacy of some epigenetic modulators like S-Adenosyl methionine (SAM), S- Adenosyl homocysteine (SAH) on breast cancer cell lines.

Notch signaling is an important embryonic signaling for development and tissue homeostasis and is deregulated in many human cancers. It is an important pathway for cell fate determination, stem cell maintenance and the initiation of differentiation in many tissues (Ye et al., 2012). Notch was discovered first in Drosophila melanogaster nearly a century ago pioneered the way to an ever-widening understanding of notch regulated or notch influenced cellular processes. The deregulation of such a pleiotropic pathway leads to several pathological conditions including cancer. Deregulated Notch signaling is well established in haematological malignancies and more recent studies have proved the importance of Notch activity in solid tumors. As the recent findings suggests it as an oncogene in some cancers but as a tumor suppressor in others. The role of Notch in solid tumors seems to be highly context dependent (Ranganathan et al., 2011).

Notch receptors are a class of single-pass trans-membrane proteins encoded by the Notch genes which can be activated by the binding of a family of compatible ligands. Four Notch receptors have been identified till date in mammals, including human, described as Notch-1-4. The mammalian canonical notch ligands are total five in number and divided into two groups denoted as Delta-like (Delta-like 1, Delta-like 3, and Delta-like 4) or Serrate-

like ligands, known as Jagged-1 and Jagged-2. All the four Notch receptors are very similar in structure except some minute differences in their extracellular and intra-cytoplasmic domains. The fine structure of extracellular domains of Notch contains a number of repeated copies of an epidermal growth factor (EGF)-like motif that are involved in ligand interaction. The Notch-1 and Notch-2 proteins have 36 sequenced repeats of EGF-like domain and the Notch-3 and Notch-4 contain 34 and 29 EGF-like repeats respectively. In the amino-terminal the sequence of EGF-like repeats are followed by a group of cysteine rich Notch Lin12 repeats (N/Lin12) which facilitates interactions of the extracellular and the intracellular domains. In the cytoplasmic region of Notch there exists domains like a Recombination Signal-Binding Protein 1 for J-kappa (RBP-J)-association molecule (RAM) domain, ankyrin (ANK) repeats, nuclear localization signals (NLS), a trans-activation domain (TAD), and a region rich in proline, glutamine, serine, and threonine residues (PEST) sequence. It is commonly known that the ANK repeats are necessary and sufficient for Notch activity while the PEST sequence is involved in Notch protein turnover.

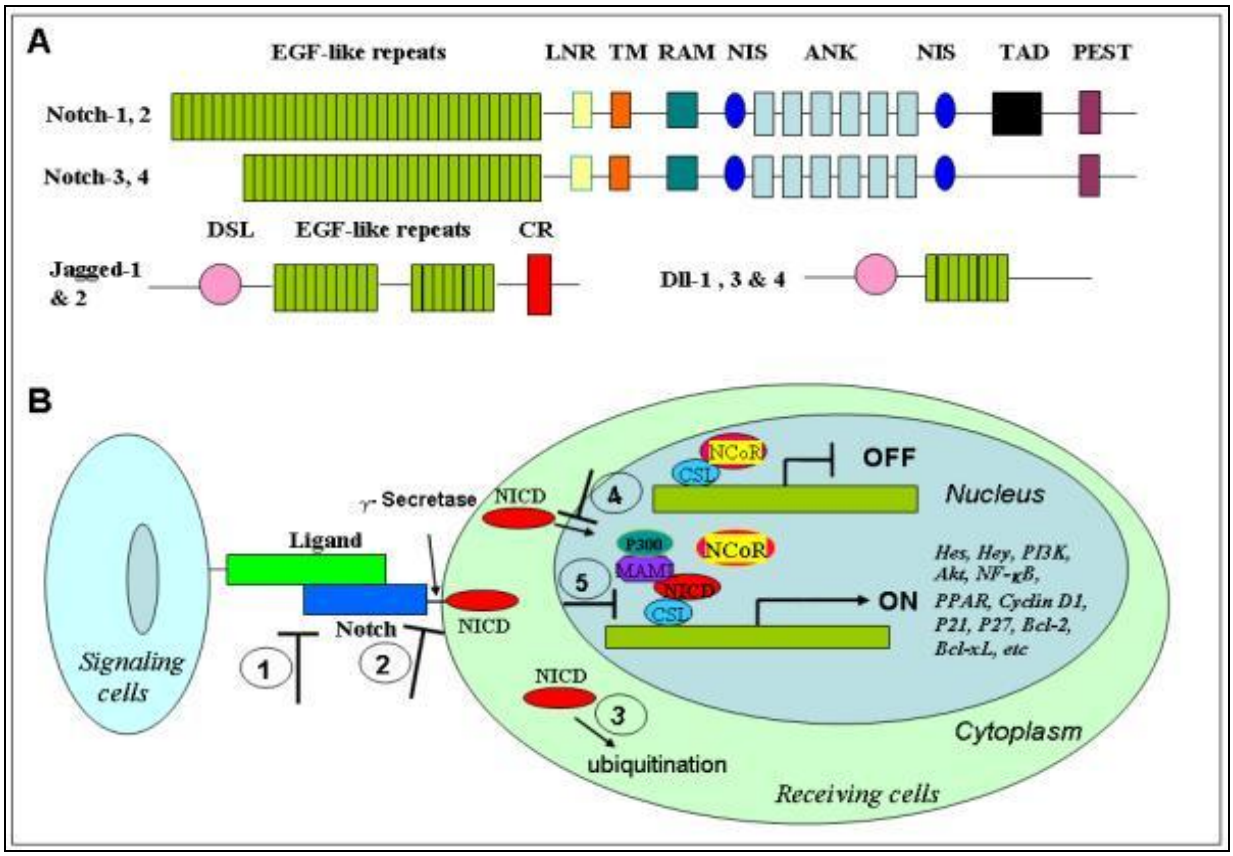

Fig.1 (A) Structure of Notch receptors (1-4) and ligands (Jagged-1, 2, Dll-1, 3, 4 (B) The Canonical notch Signaling Pathway [Figure *Ref. from, "Wang, Z., Li, Y., Kong, D., Ahmad, A., Banerjee, S., & Sarkar, F. H. (2010). Cross-talk between miRNA and Notch signaling pathways in tumor development and progression. Cancer Lett, 2009"*]

The cytoplasmic region of the receptor conveys the signal to the nucleus. The Notch ligands possess multiple EGF-like repeats in their extracellular domain and a cysteine-rich region (CR) in serrate type while these are devoid in Delta. The Jagged-1 and Jagged-2 bears almost twice the numbers of EGF-like repeats compared to Delta. Notch signaling is activated by the ligand dependent way where the neighboring cells secrete the ligand. A series of proteolytic cleavages are underway by the metalloproteases, tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase complex (comprised of presenilin-1/2, nicastrin, Pen- 2, and Aph-1) when the signaling is active. TACE sheds the first blood making the first cleavage that leads to cleave the receptor in the extracellular domain. The trans-endocytosis of the released extracellular domain takes place by the ligand expressing cell. The second cut is usually caused by the γ-secretase complex releases the Notch intracellular domain (NICD) into the cytoplasm, which can subsequently translocate into the nucleus because of the presence of nuclear localization signals located within it. Blocking the γ-secretase function prevents the cleavage of the Notch receptor thereby stopping the Notch signal transduction. The γ-secretase inhibitors (GSI) could be potential candidates for the treatment of human malignancies. When the NICD is absent the transcription of Notch target genes is blocked with a repressor complex mediated by the CSL (C protein binding factor 1/Suppressor of Hairless/Lag-1). When NICD comes in to the nucleus, it forms an active transcriptional complex by displacing the histone deacetylase–corepressor complex and recruiting the protein mastermind like1 (MAML1) and histone acetyl transferases (HAT) to the CSL complex and leads to its conversion from a transcriptional repressor into a transcription activator complex. A few important Notch target genes that have been established includes Hes (Hairy enhance of split) family, Hey (Hairy/ enhancer of spit related with YRPW motif), nuclear factor-kappa B (NF- κB), vascular growth factor receptor (VEGF), mammalian target of rapamycin (mTOR), cyclin D1, c-myc, p21, p27, Akt, etc. All of these candidates have been proved with their foul play in tumor development and progression (Wang et al., 2010).

The aberrations in Notch signaling are significantly correlated to the happening and development of many cancers along with many other developmental pathways (Kar et al., 2012). The expression levels of genes associated with the Notch signaling pathway are correlated with tumor pathology and the degree of differentiation. Notch1 and Notch2 gene expression are correlated with tumor pathology type and degree of differentiation. (Jin et al., 2012). In some tumor the tumor suppressor genes are silenced partly through deacetylation of

promoter regions and the treatment with HDAC inhibitors therefore contributes to re-expression of these genes. The whole phenomenon is known to be mediated by the notch signaling components in various solid tumors. Still an in-depth understanding of the underlying molecular mechanisms behind the mode of action of HDAC inhibitors on tumour cells remains obscure .In another mechanism the bHLH protein Hairy and Enhancer of Split-1(Hes-1), that functions as a negative regulator of transcription, is one direct and important transcriptional target of Notch signaling (Stockhausen et al., 2005).Emerging reports signifies that Notch signaling pathway is one of the convicts for the drug-resistant tumor phenotype. The down-regulation of Notch pathway could be an important therapeutic approach for induction of drug sensitivity and increased inhibition of cancer cell growth, invasion, and metastasis (Wang et al., 2010).

1.1 Epigenetics and cancer

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence. DNA methylation is one of the most commonly occurring epigenetic events in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target. Epigenetics has evolved as a rapidly developing area of research. Recent studies have shown that epigenetics plays an important role in cancer biology, viral infections, activity of mobile elements, somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation. DNA methylation is an important regulator of gene transcription, and its role in carcinogenesis has been a topic of considerable interest in the last few years. Alterations in DNA methylation are common in a variety of tumours as well as in development. Of all epigenetic modifications, hyper methylation, which represses transcription of the promoter regions of tumours suppressor genes leading to gene silencing, has been most extensively studied (Patra et al., 2008) .

DNA methylation is a covalent chemical modification, resulting in the addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring. Even though most cytosine methylation occurs in the sequence context 5'-CG-3' (also called the CpG dinucleotide), some also involves CpA and CpT dinucleotides. DNA is made up of four bases, thus there are 16 possible dinucleotide combinations that can occur. Therefore the CpG-dinucleotide should occur with a frequency of approximately 6%.However, the actual presence is only 5% to10%

of its predicted frequency. This CpG suppression may be related to the hyper mutability of methylated cytosine. The human genome is not methylated uniformly and contains regions of unmethylated segments interspersed by methylated regions. In contrast to the rest of the genome, smaller regions of DNA, called "***CpG islands"***, ranging from 0.5 to 5 kb and occurring on average every 100 kb, have distinctive properties. These regions are unmethylated, GC rich (60% to 70%), have a ratio of CpG to GpC of at least 0.6, and thus do not show any suppression of the frequency of the dinucleotide CpG. Approximately half of all the genes in humans have CpG islands and these are present on both housekeeping genes and genes with tissue specific patterns of expression. DNA methylation is brought about by a group of enzyme known as the "***DNA methyltransferases (DNMTs)"***. The DNMTs known to date are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, and DNMT3B with its isoforms (Patra et al., 2002) .

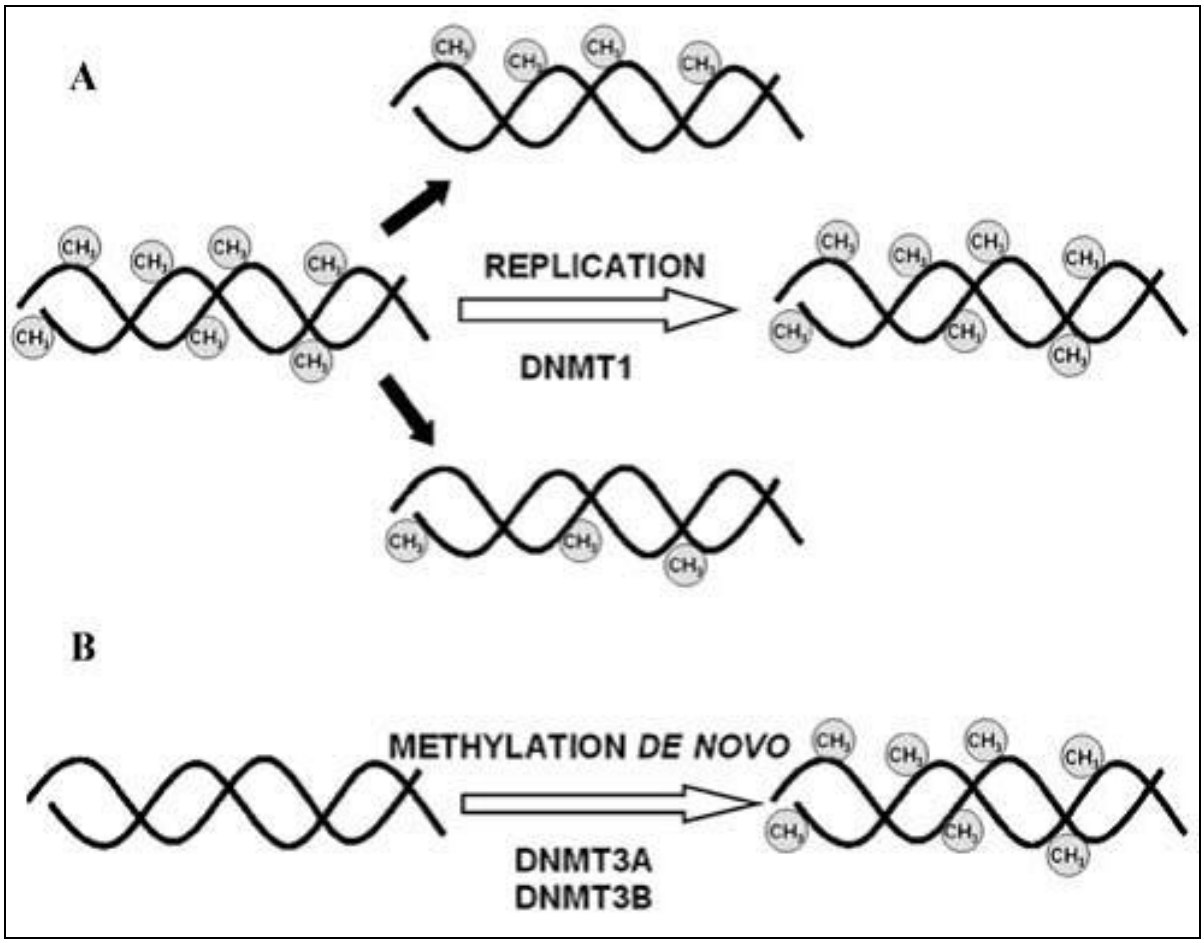

FIG 2. (A) Maintenance and (B) de novo DNMTS methylate DNA. DNMT1 binds methyl groups to the hemimethylated DNA during replication, whereas DNMT3A and DNMT3B can add methyl groups to CpG dinucleotides of unmethylated DNA.

1.1.1 DNA methylation and gene regulation

The regulation of eukaryotic gene expression is a complex process. Transcription initiation is a highly controlled and integrated event that involves *cis-acting* and *trans-acting* factors. The *cis-acting* elements are DNA sequences that act as the substrate for the trans-acting factors, and the DNA in the vicinity is prepared for transcription. Increased methylation in the promoter region of a gene leads to reduced expression, whereas methylation in the transcribed region has a variable effect on gene expression. Several mechanisms have been proposed to account for transcriptional repression by DNA methylation. The first mechanism involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters. Several transcription factors, including AP-2, c-Myc/Myn, the cyclic AMP-dependent activator CREB, E2F, and NF-kB, recognize sequences that contain CpG residues, and binding of each has been shown to be inhibited by methylation.

The second mode of repression involves a direct binding of specific transcriptional repressors to methylated DNA. The DNA methylation signals are analyzed by the methyl-CpG–binding proteins, the target being the 5-methylated CpG sequence. MeCP1 and MeCP2 were the first two protein complexes identified. However, several new proteins have now been identified. They include MBD1, MBD2, MBD4, and Kaiso. MeCP1, MBD1, MBD2, and MBD4 bind to 5mCpG through a motif called the ***methyl CpG binding domain (MBD).*** Kaiso, however, is different in mechanism, as it binds through a zinc finger motif. MBD4 is associated with DNA repair, whereas MBD1, MBD2, MeCP2, and Kaiso have been shown to repress transcription both in vitro and in cell culture assays by interacting with histone deacetylase complexes. DNA methylation can also affect histone modifications and chromatin structure, which, in turn, can alter gene expression. The underlying patterns of methylated cytosines are important in guiding histone deacetylation to certain residues. At present, there are five known proteins that have the methyl-CpG–binding domain, and four of these (MeCP2, MBD1, MBD2, and MBD3) are implicated in transcriptional repression. Three of these (MeCP2, MBD2, and MBD3) are in complexes (MeCP-2, MeCP-1 and Mi-2, respectively) that contain histone deacetylase. Studies of methylated transfected genes containing binding sites for all four of these methyl-binding proteins have shown at least partial abrogation of transcriptional repression by treatment with the histone deacetylase

inhibitor, ***Trichostatin A***. Earlier it was suggested that histone modification was secondary to DNA methylation, but recent studies on fungus revealed that histone modification can on its own commence the process of DNA methylation. The methylation of lysine in histones by specific histone methylases is also implicated in changes in chromatin structure and gene regulation. A zone of deacetylated histone H3 and methylation of histone H3 at lysine 9 surrounds a hypermethylated, silenced hMLH1 promoter, which, when unmethylated and active, is associated with acetylated H3 and methylation of histone H3 at lysine 4 position. Inhibiting DNA methyltransferases, but not histone deacetylases, leads initially to promoter demethylation, followed by gene re-expression, and finally to complete histone code reversal.

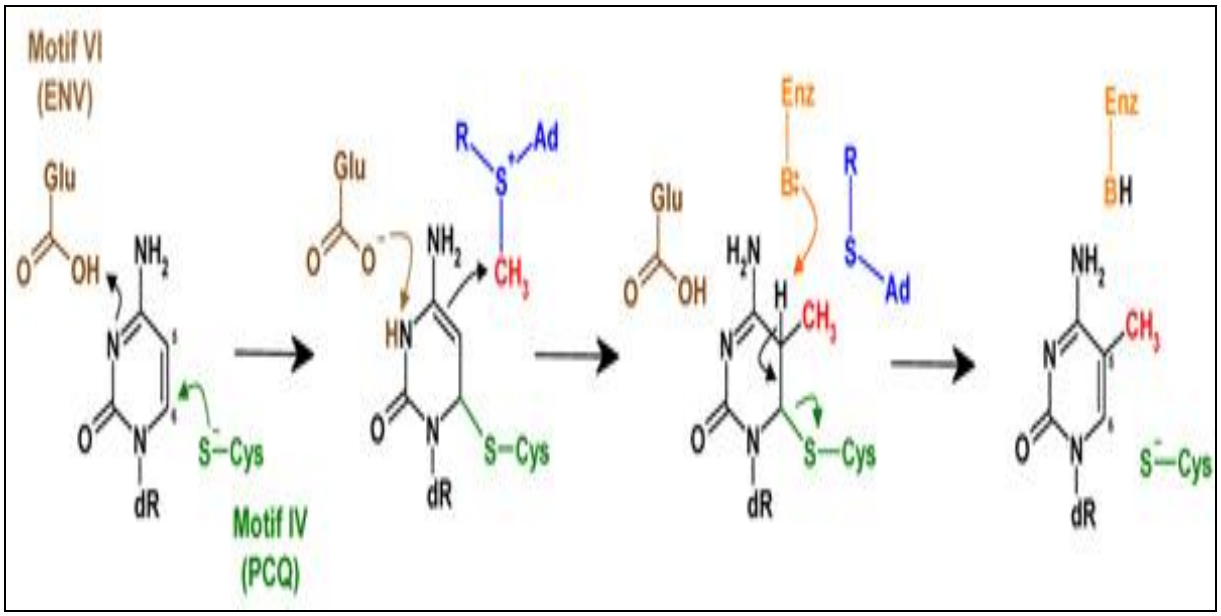

FIG 3. Mechanism of DNA methylation proposed by Reither et al. (2003) and S K Patra et al. (2008) Cancer and Metast Rev

1.1.2 Role of DNA methylation in carcinogenesis

Earlier it was thought that normal cells become progressively transformed to malignant cells as a consequence of damage to the genome, which could be a gain, loss, or mutation of the genetic information. These events cause critical loss of gene activity and thereby predispose to cancer. DNA methylation can modify the gene activity without changing the gene sequence and has been proposed as one of the two hits in "***Knudson's two hits hypothesis"*** for oncogenic transformation. Methylation changes have been implicated in tumorigenesis. DNA methylation in cancer has become the topic of intense investigation. As compared with normal cells, the malignant cells show major disruptions in their DNA

methylation patterns. ***Hypomethylation*** usually involves repeated DNA sequences, such as ***Long Interspersed Nuclear Elements (LINE)***, whereas ***Hypermethylation*** involves ***CpG islands.*** Both hypo- and hypermethylation play a prominent role in carcinogenesis, and their contribution shows scarcely defined boundaries. It has long been known that in cancer cells both alterations co-exist: malignant tumors show global hypomethylation and regional hypermethylation. Whether one must precede the other or whether both should start at the same time remains to be elucidated. In terms of carcinogenesis, the first observations in fact were done on hypomethylation (Feinberg et al., 1983); later, the discovery of regional hypermethylation as a means to silence the tumor suppressor genes expression gained the most attention (de Bustros et al., 1988).

1.1.3 Hyper-methylation and gene silencing

Observations that tumour suppressor genes can be inactivated not only through structural changes (mutation, deletion) but also by lack of expression due to promoter hypermethylation positioned tumour suppressor gene epigenetic silencing as a well-established oncogenic process (Laird et al., 1994). The first suppressor gene known to be hypermethylated and silenced was Retinoblastoma (RB) (Greger et al., 1989), which was followed by multiple publications describing similar findings for a variety of tumour suppressor genes, among them p16, MLH1, VHL, and E-cadherin (Santini et al., 2001).

To date, numerous genes have been found to undergo hypermethylation in cancer. The genes that are susceptible are the genes involved in cell cycle regulation (p16INK4a, p15INK4a, Rb, p14ARF) genes associated with DNA repair (BRCA1, MGMT), apoptosis (DAPK, TMS1), drug resistance, detoxification, differentiation, angiogenesis, and metastasis. Although certain genes such as RASSF1A and p16 are commonly methylated in a variety of cancers, other genes are methylated in specific cancers. One example is the GSTP1 gene, which is hypermethylated in more than 90% of prostate cancers but is largely unmethylated in acute myeloid leukaemia (Lee et al., 1994; Melki et al., 1999). The mechanisms involved in targeting of methylation to specific genes in cancer remain to be determined. In one report, the leukaemia-promoting PMLRAR fusion protein induced gene hypermethylation and silencing by recruiting DNA methyltransferases to target promoters (Di Croce et al., 2002). Interestingly, retinoic acid treatment induced promoter demethylation, gene re-expression, and reversion of the transformed phenotype. Many tumors show some kind of

hypermethylation of one or more genes. One of the most detailed studies was conducted on lung cancer, and more than 40 genes were found to have some degree of alteration in DNA methylation patterns. Of the various genes studied, the commonly hypermethylated ones include RARβ2, RASSF1A, CDNK2A, CHD13, and APC (Tsou et al., 2002). Hypermethylation results in loss of expression of a variety of genes critical in the development of breast cancer. These include steroid receptor genes, cell adhesion genes, and inhibitors of matrix metalloproteinases (Yang et al., 2001). Among the genes commonly hypermethylated in breast cancer are the p16NK4A, estrogen receptor (ER) alpha, the progesterone receptor (PR), BRCA1, GSTP1, TIMP-3, and E-cadherin. The steroid receptor genes, ER and PR, have long been associated with breast cancer. Methylation studies of these have shown that the ER gene has a CpG island in its promoter and first exon areas (Yang et al., 2001). The ER gene is unmethylated in normal cells and in ER-positive cell lines but shows a high degree of methylation in more than half of primary cancers. The BRCA1 gene, located at chromosome 17q21, is one of the more commonly associated genes in breast cancer, and the protein product is reduced or absent. DNA methylation has been proposed as one of the causes of its inactivation (Catteau et al., 2002).

Whether gene promoter hypermetylation is the cause or consequence for the tumor suppressor gene silencing is still a matter of controversy; nevertheless, these views are not mutually exclusive. DNA methylation is causal has been shown by the ability of diverse pharmacologic compounds and molecular techniques to reactivate gene expression upon inhibition of DNA methylation in cancer cells (Szyf et al., 2003). On the other hand, other findings suggest that hypermethylation-induced gene silencing could be secondary to changes that determine gene expression, such as chromatin modification, so that methylation helps to maintain the silenced status of the gene. Strong support for the second view came from experiments showing that methylation of histone H3 lysine 9–that is, chromatin modification occurred, along with re-silencing of p16 in absence of DNA methylation in cells in which p16 had previously been activated by knocked out of DNA methyltransferase (Bachman, 2003) and by data demonstrating p16 silencing in mammary epithelial cells that had escaped senescence and had demethylated the promoter (Clark et al., 2002).

1.1.4 Hypomethylation and gene activation

Tumour cells have global DNA hypomethylation that can be as high as 60% less than their normal counterparts (Goelz et al., 1985). It is common in solid tumours such as metastatic hepatocellular cancer, (Lin et al., 2001) in cervical cancer, (Kim et al., 1994) prostate tumours (Bedford et al., 1987), and also in hematologic malignancies such as B-cell chronic lymphoblastic leukaemia (Ehrlich, 2002). The global hypomethylation seen in a number of cancers, such as breast, cervical, and brain, show a progressive increase with the grade of malignancy (Ehrlich, 2002). This hypomethylation occurs mainly in the body of genes (coding regions and introns), as well as in pericentromeric regions of chromosomes rich in repetitive DNA sequences (Ehrlich, 2002). Interestingly, hypomethylation is progressive from premalignant conditions to fully developed malignancies (Dunn, 2003). The main mechanisms put forward in attempting to explain cancer causation by hypomethylation include chromosome instability and reactivation of transposable elements and/or inappropriate gene activation (Gamma-Sosa et al., 1983). (Oncogenes such as cMYC and H-RAS75). There are two pieces of convincing evidence linking hypomethylation with chromosomal instability. The congenital disorder ICFs syndrome immunodeficiency, chromosomal instability, and facial anomalies caused by mutations at DNMT3b demonstrates loss of methylation in classical satellite DNA and mitogen-inducible formation of bizarre multiracial chromosomes that contain arms from chromosomes 1 and 16 (Eden A et al., 2003). This disorder, however, is not associated with cancer, but common somatic tumors such as breast, ovarian, and other epithelial tumors commonly have unbalanced chromosomal translocations with breakpoints in the pericentromeric DNA of chromosomes 1 and 16 (Hansen RS et al., 1999). In mouse models with an inactivated allele of NF1 and p53 genes, introduction of a hypomorphic DNMT1 allele caused a 2.2-fold increase in LOH frequency (Narayan A et al., 1998). Finally, some reports have stressed the fact that many CpG islands are normally methylated in somatic tissues (Strichman-Almashanu LZ, 2002), and that demethylation could lead to activation of nearby genes such as HRAS. Indeed, experimental demonstration exists that hypomethylation leads to activation of genes important for cancer development, including promoter CpG demethylation and overexpression of 14-3-3sigma, maspin, heparanase, and S100A4 in several tumor types (Ogishima T et al., 2005; Sato N et al., 2003; Akiyama Y et al., 2003).

The question here is whether over-expression was indeed caused by hypomethylation or whether promoters are hypomethylated secondary to its high transcriptional activity. There are data showing that the sole hypomethylation as achieved by pharmacologic means is not sufficient to activate gene expression. In this context, some genes are not permisive for expression; this means that despite the fact that methylation is relieved the necessary ancillary factors to activate transcription are not present. Others are permissive and therefore reactivated by demethylation, whereas for others hypomethylation does not affect their levels of expression but can be overexpressed due to activation of signalling pathways known to activate them (Karpf AR et al., 2004).

1.1.5 Histone Methylation and Cancer

The transfer of methyl group from its cofactor SAM (S Adenosyl Methionine) to the amino acids basically arginine and lysine of histone protein is called as Methylation. Methylation occurs in two levels that is in the DNA level and histone level and therefore known as DNA Methylation and Histone Methylation. The enzymes those are involved in DNA Methylation are DNMT (DNA Methyltransferase) and histone Methylation is HMT (Histone Methyltransferase). Methylation works as gene silencing as a result of which they repress the transcription process. Methylation in addition is of two types i.e. genome wide hypomethylation and regional hypermethylation. Histone methylation takes place at lysine and arginine residues. Methylation of lysine takes place at H3K4, H3K36, H3K79, H3K9, H3K27 and H4K20 position. Arginine methylation of histone takes place at H3R2, H3R17 and H3R26 etc.

2. Epigenetic Modulators: SAM and SAH

2.1 S-Adenosyl Methionine (SAM)

S-Adenosyl methionine (ademetionine, AdoMet, SAM, SAMe, SAM-e) is a common co-substrate involved in methyl group transfers. SAM was first discovered in Italy by G. L. Cantoni in 1952. It is made from adenosine triphosphate (ATP) and methionine by methionine adenosyltransferase (EC 2.5.1.6). Transmethylation, trans-sulfuration, and aminopropylation are the metabolic pathways that use SAM. Although these anabolic reactions occur throughout the body, most SAM is produced and consumed in the liver. The methyl group (CH_3) attached to the methionine sulfur atom in SAM is chemically reactive. This allows donation of this group to an acceptor substrate in trans

methylation reactions. More than 40 metabolic reactions involve the transfer of a methyl group from SAM to various substrates, such as nucleic acids, proteins, lipids and secondary metabolites. In bacteria, SAM is bound by the SAM riboswitch which regulates genes involved in methionine or cysteine biosynthesis.

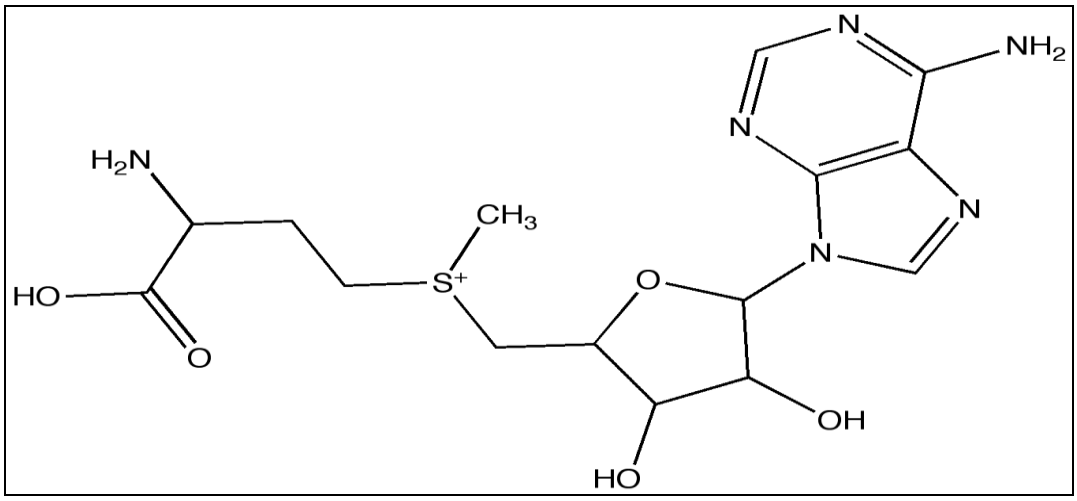

Fig 4. Molecular structure of S-Adenosyl Methionine (SAM)

2.2 S-Adenosyl Homocysteine

S-Adenosyl-L-homocysteine (SAH) is an amino acid derivative used in several metabolic pathways in most organisms. It is an intermediate in the synthesis of cysteine and adenosine. SAH is formed by the demethylation of S-adenosyl-L-methionine (SAM).

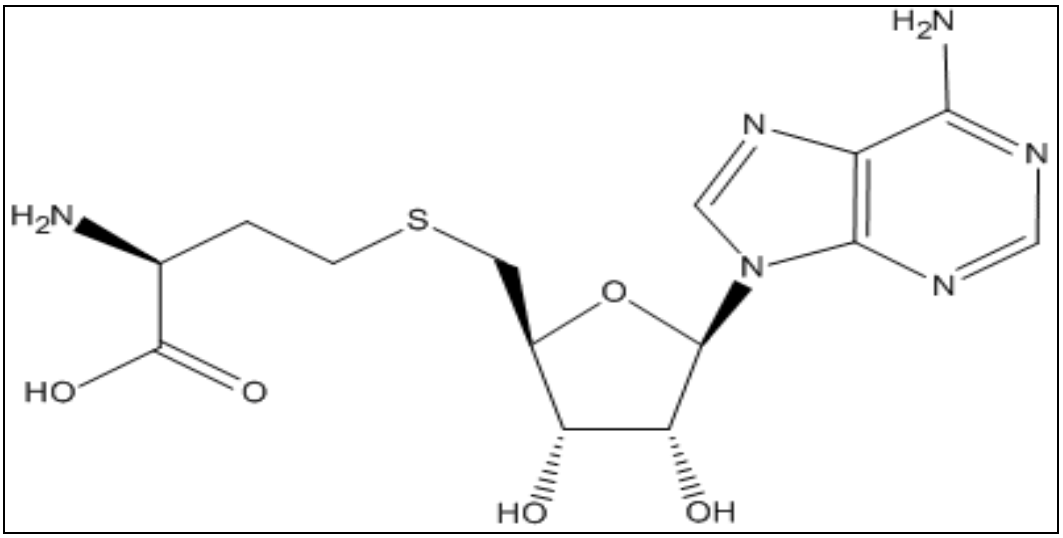

Fig 5. Molecular structure of S-Adenosyl Homocysteine (SAH)

REVIEW OF LITERATURE

1. Notch signaling in cancers

The expression of the first two Notch receptors Notch1 and Notch2 have important role in tumor pathology, type casting and degree of differentiation. In the tumors expressing high levels of Notch2 and JAG1 are correlated with a significantly better prognosis in comparison to patients expressing stunted levels of Notch2 and JAG1 (Jin et al., 2012).Involvement of Notch signaling is variedly reported in different cancers.

Notch expression is not yet fully determined in hepatoblastoma as most of the genes closed to Notch signaling pathway show abridged expression. But in another study of hepatoblastoma, NOTCH1 along with DLL1, CD44, FZD2, GLI1, IL17B, LMO2, LOR, PAX5, PT-CRA, SH2D1A and WISP1 were shown to be upregulated and contributed to invasiveness but DLL1, HEY1, DTX1, HDAC1, NOTCH2 and JAG1 were not found to be methylated there (Aktas et al., 2010). Within Kaposisarcoma (KS) lesions, the KSHV-infected spindle cells showed features compatible with KSHV-induced EndMT and involves a complex phenotype of endothelial and mesenchymal properties with Notch activity, and nuclear ZEB1 expression. This proves Notch signaling inhibitors could be effective in KS treatment (Gasperini et al., 2012). The active role of Notch in HPV-mediated transformations in different gynecological cancers is dose dependent and correlated to a variation in activator protein, AP-1 expression (Henken et al., 2012). Delta-like ligand 4 (DLL4) is one of the major notch inducing ligand and its expression is vividly associated with poor prognosis for surgically resected Pancreatic ductal adeno-carcinoma (PDAC), advanced tumor stage and lymph node metastasis (Chen et al., 2012). In oral squamous cell carcinoma tumor growth is promoted by Notch-1 along with β-catenin (component of Wnt signaling), ΔNp63 (proliferation marker and trans-membrane receptor) expression. The same study also aims to investigate the interaction between β-catenin and ΔNp63 in oral cancer(Ravindran and Devaraj, 2012). The down regulated expression of Notch 1 was related to invasion and differentiation in oral carcinoma cells. In some lingual squamous carcinoma cell Notch1 down-regulation could be correlated with the expression of matrix metallo-proteinase (MMP)-2 and MMP-9 to promote inhibition of invasion and metastasis (Yu et al., 2012a).

ER-Notch signaling along with the GPR30-PI3K/AKT signaling mediates regulation of proliferation in nuclear ER-positive endometrial cancer cells (Wei et al., 2012). The down-regulation of Notch3 sufficiently forces Rhabdomyosarcoma (RMS) cells to complete a correct full myogenic program thus provides evidence that it contributes to their malignant phenotype partially through HES1 sustained expression, (Raimondi et al., 2012). Deregulation of NUMB-mediated suppression of NOTCH1 by TNFα/IKKα- associated FOXA2 inhibition likely contributes to inflammation-mediated cancer pathogenesis in hepatocellular carcinoma (Liu et al., 2012). Notch2 and JAG1 expression levels are correlated with survival in colorectal cancers (Jin et al., 2012). Kaposi's sarcoma-associated herpes-virus (KSHV) activated Notch-induced transcription factors Slug and ZEB1, and canonical Notch signaling is pivotal for KSHV-induced Endothelial-mesenchymal Transition (EndMT). So Notch inhibitors together with the currently used chemotherapeutic drugs to improve the outcome of Osteosarcoma (OS) treatment (Ma et al., 2013). The link between the anti-apoptotic pathways of Notch, Wnt and Hedgehog and bone marrow stromal cells in chronic lymphoblastic leukemia (CLL) has been pointed out only recently. A number of anti-cancer drugs, including γ-secretase inhibitors, Cyclopamine and Quercetin, were reported to block Notch along with Wnt, and Hedgehog anti-apoptotic signaling pathways respectively in these cancers (Seke Etet et al., 2012). Overexpressed DLL4 on neoplastic cells is shown to enhance chemo resistance via angiogenesis-dependent/independent mechanisms in pancreatic ductal adeno-carcinoma PDAC(Kang et al., 2013). The matrix cellular protein, secreted protein acidic and rich in cysteine (SPARC), is known to inhibit proliferation and migration of endothelial cells stimulated by growth factors. In a recent report pSPARCCM- suppressed expression of growth factors was shown to be mediated by inhibition of the Notch signaling pathway, and cells cultured on conditioned medium from tumor cells overexpress both Notch intracellular domain (NICD-CM) and SPARC resumed the pSPARCCM- suppressed capillary tube formation and growth factor expression in vitro (Gorantla et al., 2013).

Notch signaling aggravates the efficacy of some drugs in some cancers and also is activated by some drugs and in many ways can contribute to targeted cancer therapy. Notch2 activation impedes inhibitory effect of Benzene isothiocyanate (BITC) on cell migration in human breast cancer cells (Kim et al., 2012). Withaferin A (WA) one small-molecule constituent of the ayurvedic medicine plant *Withania somnifera* with efficacy against cultured and xenografted human breast cancer cells treatment activates Notch2 and Notch4, which impede inhibitory effect of WA on breast cancer cell migration (Lee et al., 2012).

Organization of tumor cells into capillary like structures (CLS) is known as vasiculogenic mimicry (VM). VM regulated by Notch signaling may present a novel target in melanoma therapy (Vartanian et al., 2012). Curcumin is a potent inhibitor of esophageal cancer growth that targets the Notch-1 activating gamma-secretase complex proteins. This suggests that Notch signaling is pivotal in oesopageal cancer growth and its inhibition is a novel mechanism of action for curcumin during therapeutic intervention in esophageal cancers (Subramaniam et al., 2012). The expression of DLL4 is associated with reduced radio-resistance, presumably by reducing hypoxia and improving chemotherapy accessibility. Using the combination of CD31 and DLL4 staining, a classification is suggested so that HNSCCs are categorized in sub-groups to be targeted by different anti-angiogenic and hypoxia targeting agents (Koukourakis et al., 2012). In human myeloma cells, RT-PCR and flow-cytometry analysis has revealed that JAG2, the major Notch ligand, is often expressed by CD138+ primary cells and recent out comes indicate that spontaneous clonogenic growth of myeloma cells requires the expression of JAG2 (Chiron et al., 2012).

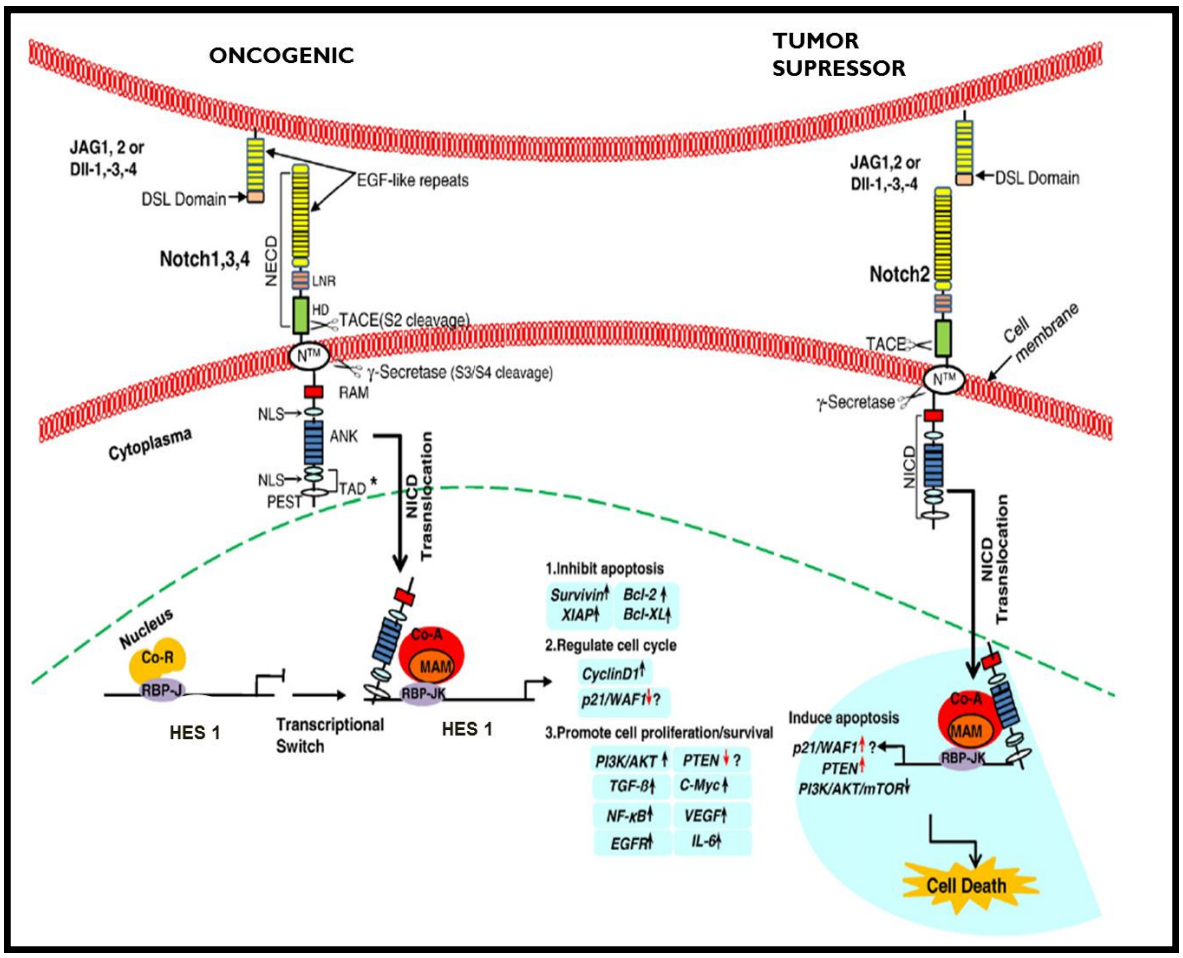

Fig 6. Aberrant Notch signaling in cancers showing context dependent properties

Correlation of Notch1, 2, 3, Jagged1, cMET, and pMAPK, has shown their correlation to clinicopathologic characteristics and prognostic utility for prediction of patient outcome (Krikelis et al., 2012).The Notch repressor Numbl negatively regulates glioma cell migration and invasion by abrogating TNF receptor-associated factor 5, TRAF5-induced activation of NF-κB this suggests that Notch inhibitors may be best used in combination with other agents or therapy. (Yu et al., 2012b). Down-regulated Notch1 is shown to be an effective approach to inactivating Snail/E-cadherin by regulating the COX-2 enzyme responsible for inflammation and pain results in inhibition of the invasion and migration of Hepatocellular carcinoma cells (HCC) cells. Here the inhibitory effects of down regulated Notch1 on cell invasion and migration were found to be independent of apoptosis (Zhou et al., 2012). In Nasopharyngeal carcinoma (NPC) patients high expression level of DLL4 ligand serves as an independent predictor of poor prognosis (Zhang et al., 2013). Notch has an important role in lung cancer and treatment with GSI after radiation can significantly enhance radiation mediated tumor cytotoxicity (Mizugaki et al., 2012).

Aberrant Notch-Hey1 signaling contributes to Embryonal rhabdomyosarcoma (ERMS) by reducing differentiation and promoting proliferation. The efficacy of Notch pathway inhibition in vivo supports the development of Notch-Hey1 axis inhibitors in the treatment of ERMS (Belyea et al., 2011). The NOTCH Modulator LNX2 overexpression and chromosome 13 amplification therefore constitutively activates the WNT pathway and offers evidence of an aberrant NOTCH-WNT axis in Colorectal cancer (CRC) progression (Camps et al., 2013). Midkine (MK), a heparin-binding growth factor that is actively overexpressed in chemoresistant Pancreatic ductal adenocarcinoma (PDAC) interacts with Notch-2 and is shown to activate Notch signaling, induced EMT, upregulated NF-kβ, and increased chemo resistance (Gungor et al., 2011). Gain-of-function mutations in Notch-1 are very common in T-cell lymphoblastic leukemia (T-ALL). The proliferation inhibitory and apoptotic effects resulting from down-regulation of Notch-1 was expected to be mediated through regulating the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 and the activity of Akt signaling in T-cell lymphoblastic leukemia (T-ALL) (Guo et al., 2009). JAG2, a notch ligand, overexpression may be an early event in the pathogenesis of multiple myeloma involving IL-6 production (Houde et al., 2004). In ovarian cancer Dll4 plays a functionally important role in both the tumor and endothelial compartments and targeting Dll4 in combination with anti-VEGF treatment can promote outcomes of treatment (Hu et al., 2011).

In another study activating point mutations in NOTCH1 in more than 50% of T-cell acute lymphoblastic leukemia (T-ALL) shows that NOTCH1 cascade as a central player of T-ALL pathogenesis (Koch and Radtke, 2011). NOTCH signaling is now shown to be pivotal in the pathogenesis of a subset of intracranial ependymomas (Pfister and Witt, 2009). Neuralized (Neurl) is a highly conserved E3 ubiquitin ligase, which in Drosophila acts upon Notch ligands to regulate Notch pathway signaling. Human Neuralized1 (NEURL1) was investigated as a potential tumor suppressor in medulloblastoma (MB). NEURL1 is a candidate tumor suppressor in MB, at least in part through its effects on the Notch pathway (Teider et al., 2010). Jagged1 may be involved in the differentiation and proliferation of tongue cancer. Targeting Jagged1 RNA interference by lentiviral vector can effectively lower Jagged1 mRNA and protein expression levels in Tca8113 tongue cancer cell lines, thereby preventing the proliferation of Squamous cell carcinoma of the tongue (TSCC) cells (Zhang et al., 2012). Skeletrophin (mindbomb homolog 2 (MIB2)) is a RING (Really Interesting New Gene) finger-dependent ubiquitin ligase, which targets the intracellular region of Notch ligands. Exogenously expressed skeletrophin, but not its RING mutant, increased transcription of Hes1 gene, a downstream effector of Notch pathway in melanoma cells (Stockhausen et al., 2005). Ectopic Myc expression plays a key role in human tumorigenesis and Notch signaling downstream of Myc enables cells to adapt in the tumor hypoxic microenvironment (Yustein et al., 2010) .

2. Notch, Epigenetics and Cancer

Cancer is both a genetic and an epigenetic disease. Inactivation of tumor-suppressor genes by epigenetic changes is frequently observed in human cancers, particularly as a result of the modifications of histones and DNA methylation (Ferres-Marco et al., 2006). Epigenetic silencers and Notch collaborate to promote malignant tumors by Retinoblastoma (Rb) silencing (Ferres-Marco et al., 2006). Two Polycomb group epigenetic silencers, Pipsqueak and Lola that participate in this process tumorigenesis were studied in the drosophila eye. When coupled with overexpression of Delta, relating to the Notch-Delta pathway, deregulation of the expression of Pipsqueak and Lola induces the formation of metastatic tumors. This phenotype depends on the histone-modifying enzymes Rpd3 (a histone deacetylase), Su(var)3-9 and E(z), as well as on the chromodomain protein Polycomb. Expression of the gene Retinoblastoma-family protein (Rbf) is downregulated in these tumors and, indeed, this downregulation is associated with DNA hypermethylation (Ferres-Marco et

al., 2006). Neuroblastoma cell lines express multiple Notch receptors, which are inactive at baseline. Activation of the Notch pathway via ligand binding consistently resulted in growth arrest. Hes gene expression here appears to be regulated epigenetically and could be induced with decitabine. These findings support a tumor suppressor role for Notch signaling in neuroblastoma and also prove its context dependent behavior. In many childhood acute leukemias, transcription factors are altered through chromosomal translocations that change their functional properties resulting in repressed activity or inappropriate activation. Histone deacetylase inhibitors, drugs targeting the NOTCH pathway, and short interfering RNAs have shown encouraging results in pre-clinical studies and are likely to enter the clinical arena in the near future. Through an improved understanding of the pathways and mechanisms underlying the malignant transformation induced by altered transcription factors, new targeted epigenetic therapies will be designed that should greatly enhance current available treatments (Berman and Look, 2007).

The Groucho homologue Transducin-like Enhancer of Split 1 (TLE1) is a transcriptional corepressor which acts ill in the acute myelogenous leukemia through Wnt, and Notch signaling pathways. The epigenetic inactivation TLE1 contributes significantly to the development of the hematologic malignancies by disrupting important pathway of differentiation and growth-suppression (Fraga et al., 2008). Dynamic regulation of histone modifications is critical during development, and aberrant activity of chromatin-modifying enzymes has been associated with diseases such as cancer. The functional interplay between histone demethylases in vivo, providing insights into the epigenetic regulation of heterochromatin/euchromatin boundaries by Lid and dLsd1 and shows their involvement in Notch pathway-specific control of gene expression in euchromatin (Di Stefano et al., 2011). Correlation between constitutive acetylation of the JAG2 core promoter in the MM cell lines reduced levels of the SMRT corepressor that recruits HDAC to promoter regions. SMRT function restoration induced JAG2 down-regulation as well as Multiple myeloma (MM) cell apoptosis (Ghoshal et al., 2009).The miR-181c may be silenced through methylation and play important roles in gastric carcinogenesis through its target genes, such as NOTCH4 and KRAS (Hashimoto et al., 2010). Notch pathway is important in regulating osteosarcoma metastasis and may be useful as a epigenetic therapeutic target of valproic acid and other HDAC inhibitors (Hughes, 2009). Epigenetic alterations like DNA methylation and miRNAs mediated control of gene expression of multiple Notch target genes and pathway interacting genes (PPARG, CCND1, and RUNX1) may relate to activation of this pathway and poor

survival of patients with high-grade serous ovarian cancer (HGS-OvCa) (Ivan et al., 2013). Epigenetic deregulation of NOTCH4 signaling in OSCC was also observed, as part of a possible methylation signature for recurrence, with parallels to recently discovered NOTCH mutations in HNSCC (Jithesh et al., 2013).

Notch3 is acetylated and deacetylated at lysines 1692 and 1731 by p300 and HDAC1, respectively, a balance impaired by HDAC inhibitors (HDACi) that favor hyperacetylation in leukemias. Notch signaling control in which Notch3 acetylation/deacetylation process represents a key regulatory switch and also a suitable druggable target for Notch3-sustained T-cell acute lymphoblastic leukemia therapy (Palermo et al., 2012). Notch1 activity in gastric cancer is controlled by the epigenetic silencing of the ligand DLL1, and that Notch1 inhibition is associated with the diffuse type of gastric cancer (Piazzi et al., 2011). In colorectal cancer CRC cell lines, unlike gastric cancer, DLL1 expression is not regulated by promoter methylation (Piazzi et al., 2012). Histone deacetylase inhibitor valproic acid is seen to be effective by acting on Notch signaling in human neuroblastoma cells.

OBJECTIVES

The Objectives of our study were:

1. Determination of the effects of S-Adenosyl Methionine (SAM) and S-Adenosyl Homocysteine (SAH) on the cellular morphology of breast cancer cell line.

2. Role of SAM and SAH in modulation of Notch signaling pathway components (Notch-1 and HES-1) in breast cancer.

3. Correlation of Notch signaling components (Notch-1 and HES-1) expression and its role in promoting breast cancer cell death.

MATERIALS AND METHODS

1. Cell lines and culture

We obtained the MDA MB-231 cell line from the *National Centre for Cell Science (NCCS), Pune, India.* The cells are known to be of epithelial breast adenocarcinoma origin and are triple negative. These were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin-streptomycin sulfate (Invitrogen). A treatment was carried out with *S-Adenosyl methionine (SAM)* and *S-Adenosyl-L-homocysteine (SAH),* all purchased from Sigma and added to the regular growth media under sterile conditions for 3 days. The media was changed every 24 well culture plates.

2. MTT Assay

To determine the proliferative activity, MDA MB-231 cells were seeded in two 96-well plates at a density (5000-40,000 cells or 10^6 cells) based on the doubling time, with 200 µl growth media (10% FBS) and incubate for 24 hrs in incubator with 5% CO_2 concentration at 37^oC. Cell seeding must be uniform in order to obtain a dose response effect of the drug. The drug of interests S-Adenosyl Methionine (SAM) and S-Adenosyl Homocysteine (SAH) were diluted at 10 different concentrations in the growth media. In parallel the cells with the solvent control was also treated to assess its effect on cells. After 24 hours existing media was removed and replaced with media with various concentration of drugs and was incubated for 24 or 48 hours at 37^o C. To detect the cell viability MTT working solution was prepared by diluting the stock solution (stock 5mg/ml PBS, PH 7.2) in growth medium without FBS to the final concentration of 0.8mg/ml. 100 µl of MTT working solution was added to each well and incubated for 4 hours in CO_2 incubator. After incubation, the media was removed carefully without disturbing *formazan* precipitate and dissolved in 100 µl of 100% DMSO. An incubation of 15 minutes was carried out in dark and the colorimetric estimation of formazan product was performed at 570nm in a microplate reader. The data was plotted against drug concentration and non-linear regression curve fitting was performed using *graph pad prism* software to calculate the optimal growth inhibitory concentration (LC_{50}) of the drugs.

3. Isolation of Total Cellular RNA

The total cellular RNA was extracted using TRI reagent (Sigma), following the manufacturer's instructions. On the culture dish 1 ml of the TRI Reagent per 10 cm^2 of glass culture plate surface area was added. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. TRI Reagent is not compatible with plastic culture plates. To ensure complete dissociation of nucleoprotein complexes, samples were allowed to stand for 5 minutes at room temperature. 0.1 ml of 1-bromo-3-chloropropane or 0.2 ml of chloroform was added for per ml of TRI Reagent used. Samples were covered tightly, shaken vigorously for 15 seconds, and were allowed to stand for 2–15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 2–8 °C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).The aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol was added per ml of TRI Reagent used in Sample Preparation, step 1 and mixed. The sample was allowed to stand for 5–10 minutes at room temperature and centrifuged at 12,000 g for 10 minutes at 2–8 °C. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was removed and RNA pellets were washed by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent used in Sample Preparation. The sample was vortexed and then centrifuged at 7,500 ´ g for 5 minutes at 2–8 °C. The RNA pellets were briefly dried for 5–10 minutes by air drying. An appropriate volume of nuclease free water was added and mixed by repeated tapping at 25 °C for 10–15 minutes.

4. Quantification of the total cellular RNA

Final preparation of RNA was analyzed using a nano-drop UV spectrophotometric analyzer. It was likely that a standard preparation of RNA should have a 260/280 ratio of 1.7 and a 260/230 ratio of <1.65 which indicates the preparation to be free from proteins and oligo-peptides contamination. Ethidium bromide (EtBr) staining of RNA in agarose gels visualizes two predominant bands of small (2 kb) and large (5 kb) ribosomal RNA, low molecular mass (0.1–0.3 kb) RNA, and discrete bands of high molecular mass (7–15 kb) RNA.

5. cDNA Synthesis and Evaluation

In a 1.5 ml tube 5 µl of Deionized RNAse free water, 1 µl of T_{18}- oligo (1 µg), 6 µl of dNTPs (10mM), 6 µl of Total RNA (3 µg) were added to make a total volume of 18 µl . The tube with the contents was incubated at 65°C for 3 minutes. The tube was snap cooled on ice and 6 µl of Reverse Transcriptase buffer (5X) ,3 µl of DTT, 1 µl of Reverse Transcriptase, 1 µl of RNase inhibitor and 1 µl of RNase free water the following added to make a total volume of the reaction mix 12 µl .The tube was then snap spinned and incubated at 45 °C for 45 minutes. Again an incubation at 90 °C for 5 minutes followed up. After incubation a snap spin was carried out and 30 µl of deionized water was added. The cDNA prepared can now be used immediately or can be stored at -30 °C. The synthesized cDNA was evaluated by performing PCR for one of the house keeping genes such as β-actin. PCR reaction mix was prepared with 5.0 µl of 10X Taq polymerase buffer, 2.5 µl of 10mM dNTP mix, 1.5 µl of 1.5 mM $MgCl_2$, 2.0 µl of Oligo (mActin- Forward) 10 µM, 2.0 µl of Oligo (mActin- Reverse) 10 µM, 1.0 µl of Taq DNA polymerase and 26.0 µl of deionized water to make a total volume of 40.0 µl. Two aliquots of 20 µl of PCR mix was transferred into 2 PCR tubes. 5 µl of cDNA was added in one tube and 5 µl deionized water was added in another. The tubes were snap spinned to collect the contents in the bottom of the tubes and were subject to PCR with the following cycling conditions of ; 92° C for 3 minutes, 30 cycles of 92 °C 30 sec, 50 °C for 30 sec, 72 °C for 30 sec, 72 °C for 5 minutes and 4 °C for forever. The contents were loaded in 1.5% agarose gel and electrophoresed along with a molecular weight marker. The gel was visualized under UV light and results were documented in a gel documentation system.

6. RT-PCR Analysis

We used total RNA (2 µg.) isolated from the cancerous cell lines for reverse transcription and amplification. The primers used for RT-PCR were designed so that there is an intron between the amplified regions to recognize any DNA contamination. We used three sets of primers to amplify NOTCH1, HES-1 and β-actin genes having sequence as detailed in Table no. for each RNA sample. The PCR were carried out using standard protocols and the DNA was amplified under the following conditions: 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and the final extension of 72 °C for 5 min. We then analyzed the PCR products on a 1% agarose gel.

Table. 1 Primers used for RT-PCR Analysis

Serial No.	Primer Name	Sequence	Scale (uM)	Purification	Length
1	Notch1 Forward primer	5'CCGCCTTTGTGCTTCTGTT3'	0.025	DST	19
2	Notch1 Reverse primer	5'TCCTCCTCTTCCTCGCTGTT3'	0.025	DST	20
3	HES1 Forward primer	5'ATGGAGAAAAATTCCTCGTCCC3'	0.025	DST	22
4	HES1 Reverse primer	5'TTCAGAGCATCCAAAATCAGTGT3'	0.025	DST	23
5	β –ACTIN Forward	5'CTGGAACGGTGAAGGTGACA3'	0.025	DST	20
6	β -ACTIN Reverse	5'AAGGGACTTCCTGTAACAACGCA3'	0.025	DST	23

7. Study of change in cellular morphology by *Scanning Electron* Microscopy (SEM)

Cells on attaining 70% confluence on 0.2% gelatin precoated 10-mm coverslips in 35-mm petri dishes were cultured. Dishes without treatment were used as controls and were kept for the same period of induction using suitable growth media. After 24 and 48 hours of drug treatment with S-Adenosyl methionine (SAM) and S-Adenosyl-L-homocysteine (SAH), the dishes containing the coverslips with cells were used for SEM analysis. In preliminary experiments, differentiated adipocytes were detached and lipid was extracted by normal fixation and dehydration procedures used for SEM .Treatment medium from the dishes was decanted and the cells were fixed with freshly prepared 2.5% glutaraldehyde in DPBS at 4 °C for 3 h. The dishes with fixed cells were washed twice with DPBS and dehydrated with methanol. It was also found that the fixed cells could be stored in DPBS at 4 °C for a maximum of 1 week and dehydrated when necessary. Stored dishes were brought to room temperature and again washed once with DPBS. Coverslips with dishes were then dried in vacuum-assisted desiccators overnight and then stored at room temperature till SEM analysis was carried out. The surface of the coverslip was sputter-coated in a vacuum with an electrically conductive 5 nm thick layer of Platinum Precession Etching Coating system.

SEM images were then recorded with a scanning electron microscope at a lower voltage (20 kV) and low vacuum mode with a tilt of 30°.

8. Cell migration assay (Scratch assay)

60-mm dishes were coated with proper ECM substrates for the cell type to be studied by incubating the dishes overnight at 4 °C or for 2 h at 37 °C without rotation or shaking. The unbound ECM substrate was removed and dishes were blocked and coated with 3 ml of 2 mg ml-1 bovine serum albumin for 1 h at 37°C. Then the dishes were once washed with PBS and refilled with 3–5 ml of media before plating the cells. For the particular cell type used, the appropriate amount of serum in the medium during the in vitro scratch assay is required to be determined. It is always recommended to use a lower percentage of serum than that used in the growth media to minimize cell proliferation, but just sufficient to prevent apoptosis and/or cell detachment. Sub-confluent growing cells were re-suspended in a tissue culture dish by washing cells twice with PBS, adding versene containing trypsin, and then mixing cells with medium containing serum. The solution was gently pipetted and the dish was rocked to disperse the cells equally. An aliquot from the cell suspension was taken and the cell count was determined using a hemocytometer. Cells were plated onto the prepared 60-mm dish to create a confluent monolayer and incubated properly for approximately 6 hours at 37°C, allowing cells to adhere and spread on the substrate completely. The required number of cells for a confluent monolayer depends on both the particular cell type and the size of dishes and need to be adjusted appropriately. The cell monolayer was scraped in a straight line to create a ‘‘scratch’’ with a p200 pipet tip.

The debris was removed and the edge of the scratch was smoothed by washing the cells once with 1 ml of the growth medium and then replaced with 5 ml of medium specific for the in vitro scratch assay. To obtain the same field during the image acquisition markings were created to be used as reference points close to the scratch. The reference points can be made by etching the dish lightly with a razor blade on the outer bottom of the dish or with an ultrafine tip marker. After the reference points were made, the dish was placed under a phase-contrast microscope, and reference mark was left outside the capture image field but within the eye-piece field of view. The first image of the scratch was taken. The dish was placed in a tissue culture incubator at 37°C for 8–18 hours. After the incubation dish was placed under a

phase-contrast microscope, the reference point was matched; the photographed region was aligned to acquire a second image.

9. Analysis of chromatin condensation by *Hoechst 33342* stain

After treatment with drugs, the cells were stained with Hoechst 33342 stain (1 mg/ml) and incubated for 10 min at 37°C and images were taken under UV filter using Epi-fluorescent Microscope (Nikon TE 2000E). Condensed nucleus was counted against total number of nucleus in the field, and the percentages of apoptotic nuclei were analyzed.

10. Cytochemical staining of for detecting autophagosomes

An optimal number of cells were seeded based on doubling time in a 96 well plate and kept for 24 hours in incubator with 5% CO2 at 37°C. After the incubation period, the cells were challenged with the suspected autophagy modulating factor for 12 hours. Then after incubation, the existing media was removed and 100µl of fresh media containing 1µg/ml of acridine orange was added. The cells were incubated for 15min at normal culture conditions. The media was discarded and washed with PBS and fresh media was added to the cells. The cells were analyzed under a fluorescent microscope using blue filter (495nm) to view the green fluorescence (510-530nm) from free Acridine Orange and red fluorescence (> 650nm) from acidic vesicles (autophagosomes).

11. Comet assay to measure the DNA damage

Two water baths were equilibrated at 40 °C and 100 °C respectively. Than 1% low-gelling-temperature agarose was prepared by mixing powdered agarose with distilled water in a glass beaker or bottle. The bottle was placed in the 100 °C water bath for several minutes and was transferred into a 40 °C water bath. Agarose-precoated slides were prepared by dipping the slides into molten 1% agarose and wiping one side clean. It is best to work in a low-humidity environment to ensure agarose adhesion. Agarose was allowed to air-dry to a thin film. Slides can be prepared ahead of time and stored with desiccant. A single-cell suspension was prepared using enzyme disaggregation or mechanical dissociation. The cells were kept in ice-cold medium or phosphate-buffered saline to minimize cell aggregation and inhibit DNA repair. Using a hemocytometer or particle counter, cell density was adjusted to about 2 × 104

cells/ml in phosphate-buffered saline lacking divalent cations. Slides were labeled on frosted end using a pencil. 0.4 ml of cells into a 5 ml plastic disposable tube.1.2 ml 1% low-gelling-temperature agarose at was added at 40 °C.1.2 ml of cell suspension onto the agarose-covered surface of a pre-coated slide was mixed by vigorous pipetting. Agarose was allowed to be gel for about 2 min. After agarose has gelled, slides were submerged in a covered dish containing A1 lysis solution. Samples were lysed overnight (18−20 h) at 4 °C in the dark. After overnight lysis, slides were removed carefully and submerge in A2 rinse solution for 20 min at room temperature (18−25 °C). The process was repeated two times to ensure removal of salt and detergent. Care was taken for not allowing DNA to renature even briefly (i.e., by lowering pH below 12.3) until after electrophoresis, as this will result in DNA tangling and reduced migration. After these three rinses, slides were submerged in fresh A2 solution in an electrophoresis chamber. The chamber was filled with a consistent volume of buffer that is about 1–2 mm above the top of the agarose. It was ensured that the chamber is level using a bubble leveling device. Electrophoresis was conducted in solution A2 for 25 min at a voltage of 0.6 V/cm. The current was about 40 mA using 20 V. The distance in centimeters was measured between the negative and positive electrodes in the electrophoresis chamber. Slides were removed from electrophoresis chamber and were rinsed and neutralized in 400 ml of distilled water. Slides were placed in staining solution containing 2.5 μg/ml of propidium iodide in distilled water for 20 min. Finally the slides were rinsed with 400 ml distilled water to remove excess stain. Analysis of cells was done by examining at least 50 comet images from each slide. Analyzing doublets or comets at slide edges should be avoided.

RESULTS AND DISCUSSION

1. Cell viability assay by *MTT Method*

MDA MB -231 cell viability was determined after SAM and SAH treatment by MTT Assay. The IC_{50} value for both the drugs was calculated. Both the SAM and SAH treatment showed decrease in cell viability but SAM treatment showed little higher decrease in cell viability.

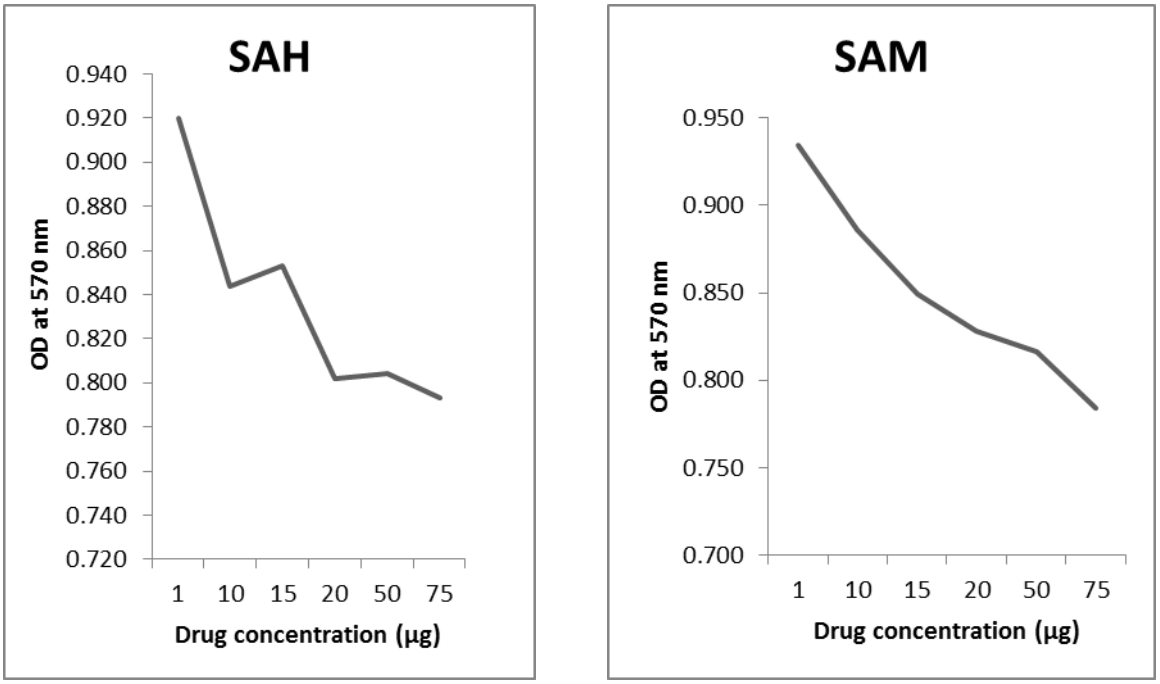

Fig 7. Graphical analysis of cell viability after SAM and SAH treatment

2. Isolation of Total Cellular RNA by *Trizol Method*

The total cellular RNA isolated was isolated following the manufacturer's instructions by Tri-reagent (Sigma).The isolation of the RNA was carried out from the cultured cells of the wells of the six well plates from two wells in a time dependent manner of 24 hours and 48 hours. The isolation was almost pure and in good yield as the reading in Nano drop spectrophotometer showed the 260/280 absorption ratio above 1.7 and the 260/230 absorption ratio above 1.65 for all the samples quantified.

3. cDNA Synthesis and *RT-PCR*

Previous studies and the RT-PCR data confirmed that Notch1 and HES1 were up regulated in breast cancer cell line MDA-MB 231. A time dependent treatment with S-Adenosyl Methionine (SAM) for 24hours and 48 hours showed down regulation of these components but similar treatment with S-Adenosyl Homocysteine (SAH) showed the up regulation of these components.

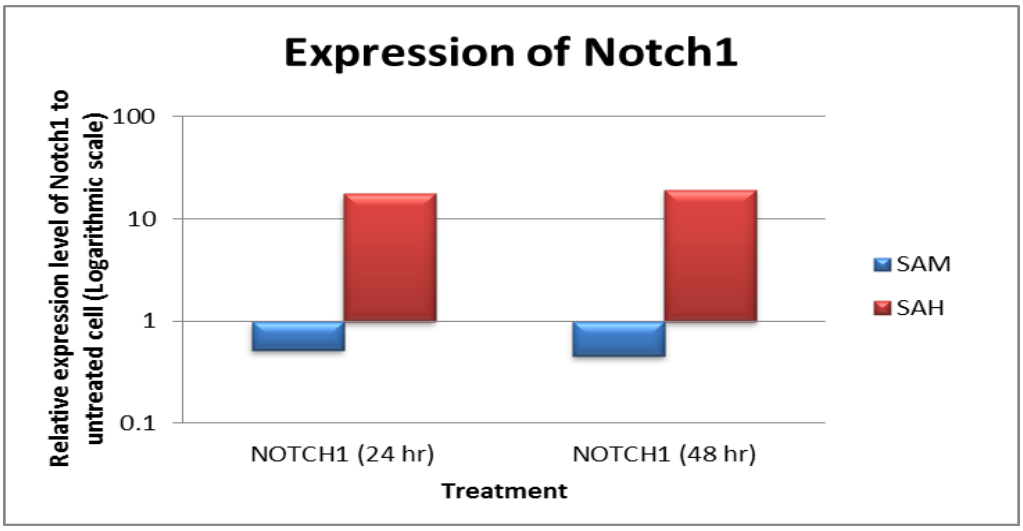

Fig 8. Graphical representation of RT-PCR results for expression level of Notch1 after SAM and SAH treatment

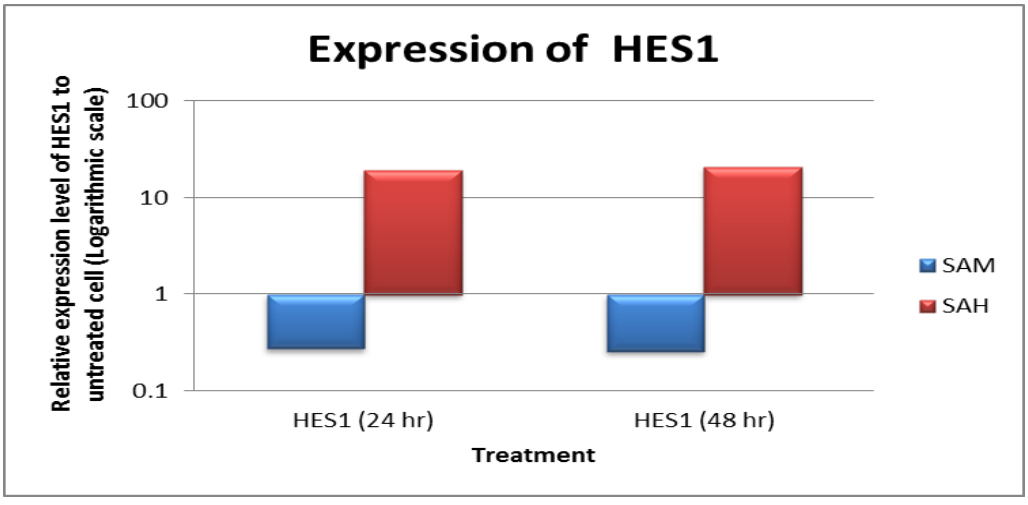

Fig 9. Graphical representation of RT-PCR results for expression level of HES1 after SAM and SAH treatment

4. Study of change in cellular morphology by *Scanning Electron Microscopy (SEM)*

After treatment of the MDA MB-231 cells with SAM and SAH in a time dependent manner for 24 and 48 hours the change in the cellular morphology was observed by a Scanning Electron Microscope. The images suggested that there was a change in the normal cellular morphology of MDA MB-231 cells as they started to attain a round shape suggesting an induction of a mechanism like apoptosis. But in the SAH treated cells there was no such significant change in the cellular morphology.

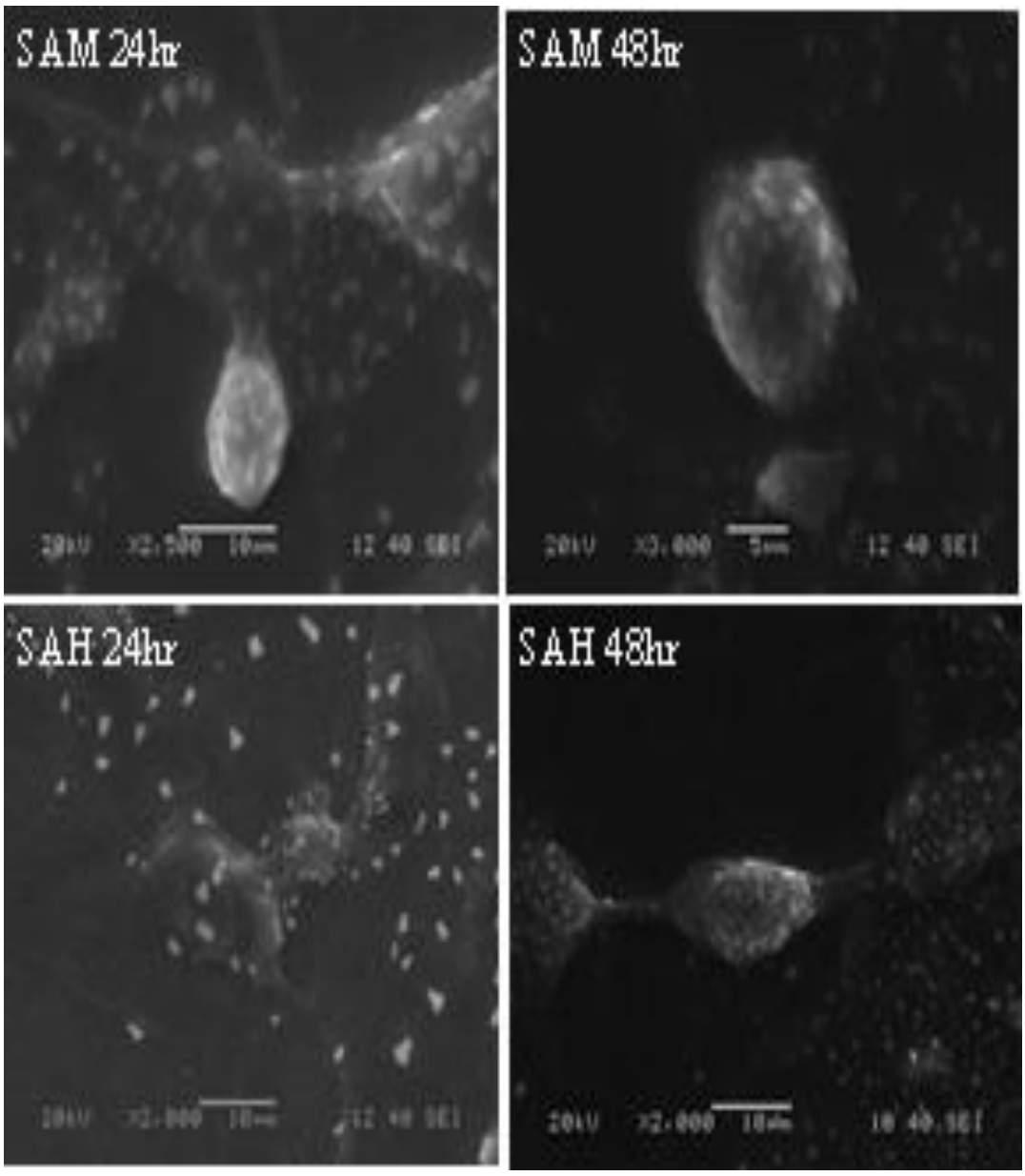

Fig 10. SEM images showing changes in cellular morphology after SAM and SAH treatment

5. Cell migration study by *Scratch assay*

For the study of migratory property of the MDA MB-231 cells after SAM and SAH treatment for 0-48hr in a time dependent manner the scratch assay was performed .The results showed that there was more migration of cells towards the scratched area in SAH treated cells as compared to the SAM treated in comparison with the untreated plates taken as control in a time dependent manner.

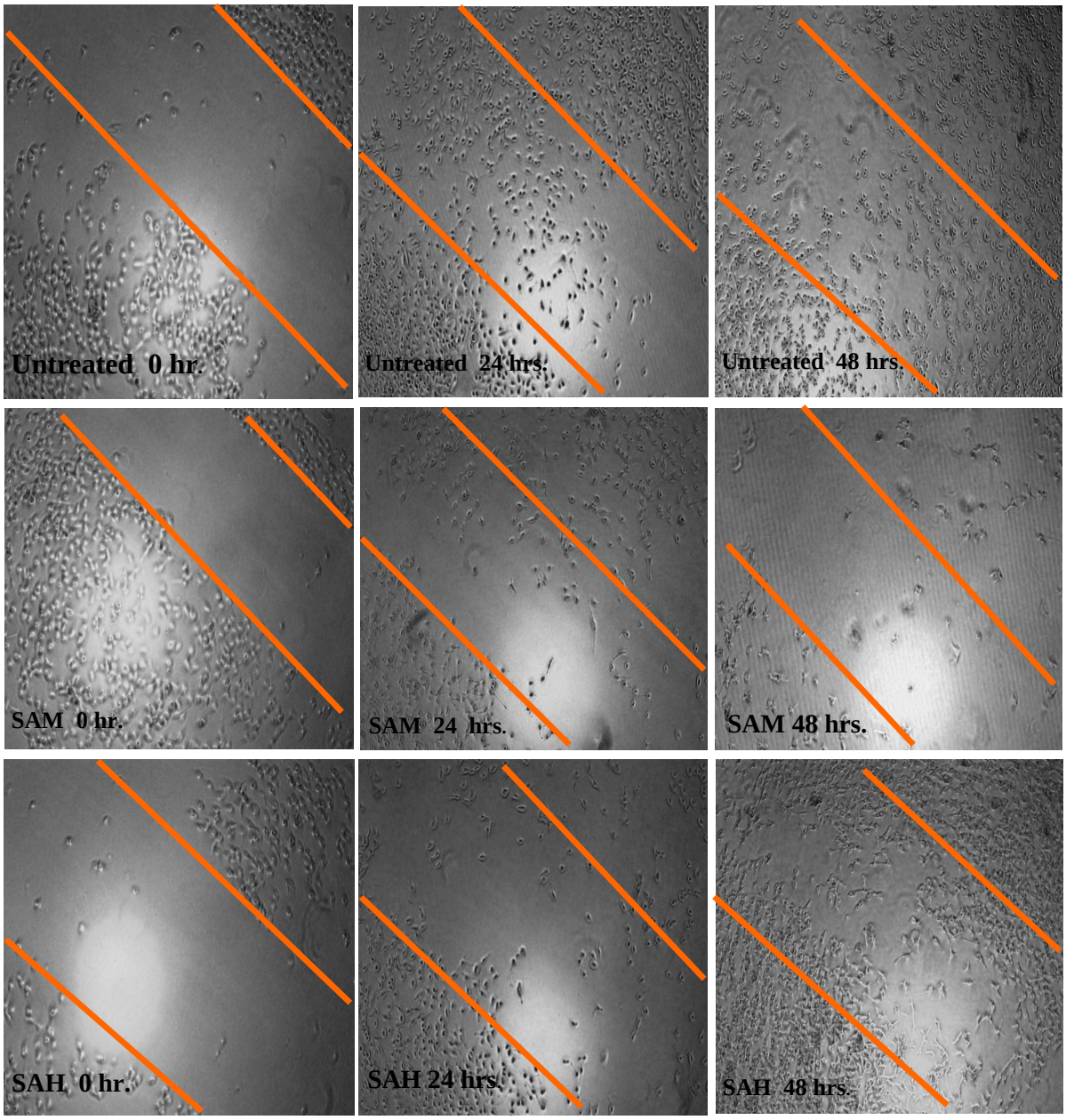

Fig 11. Microscopic images showing changes in the migratory property of MDA MB-231 cells after SAM and SAH treatment

6. Analysis of chromatin condensation by *Hoechst 33342 stain*

After treatment with SAM and SAH for 0-48hr in a time dependent manner the MDA-MB-231 cells were stained with ***Hoechst 33342 stain*** and analyzed for the chromatin condensation. The results indicated that there was formation of more condensed chromatin structures after treatment with SAM than SAH in a time dependent manner.

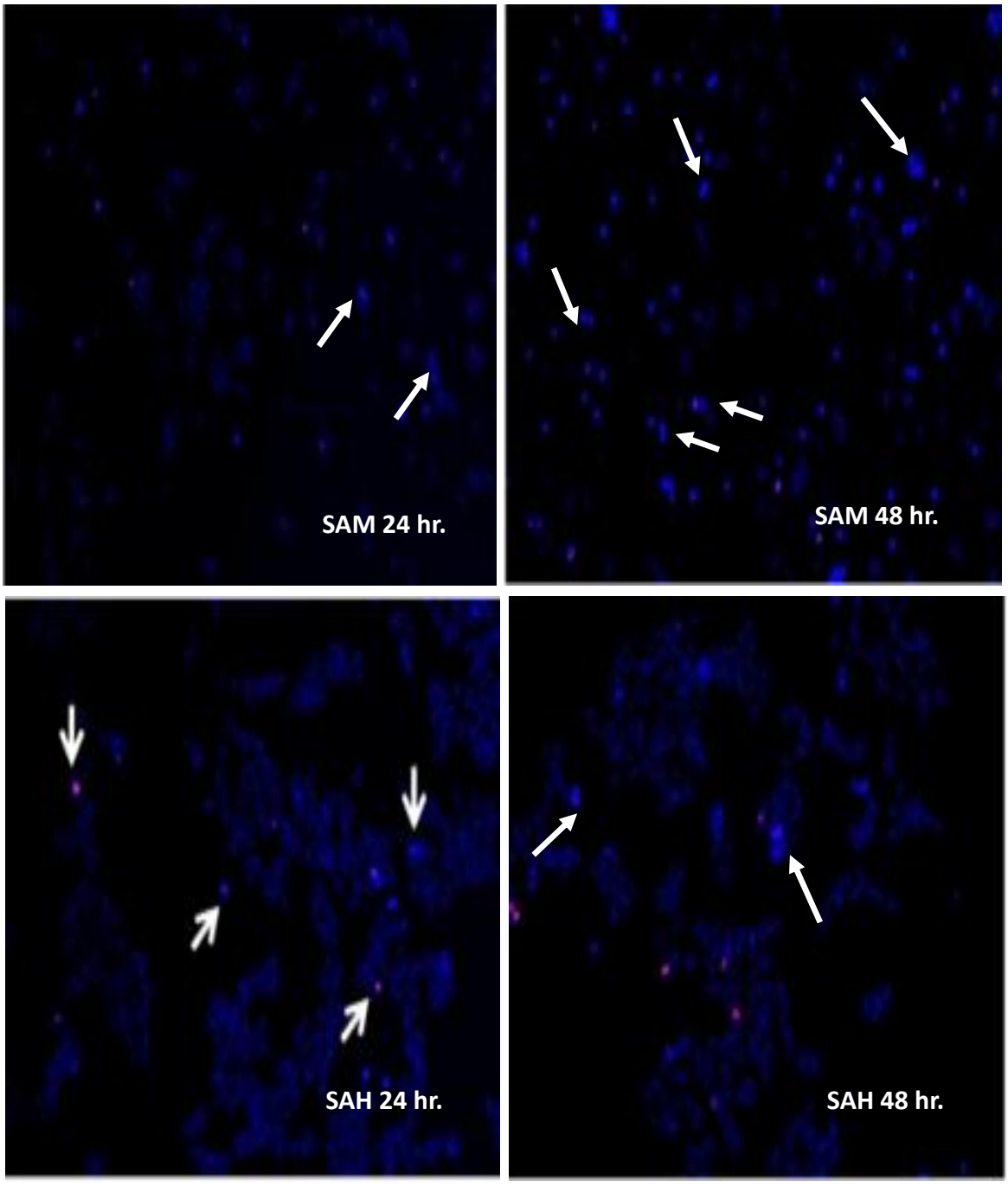

Fig 12. Fluorescence microscopic condensed chromatin images showing after SAM and SAH treatment

7. Cytochemical staining for detecting autophagosomes

The MDA MB-231 cells after the formation of the treatment with SAM and SAH for 24-48 hr. in a time dependent manner were analyzed for the formation of autophagosomes. The SAM treated cells showed greater frequency in the formation of autophagosomes after a treatment of 48 hours. But the SAH treatment was found less efficient in forming the autophagic vesicles.

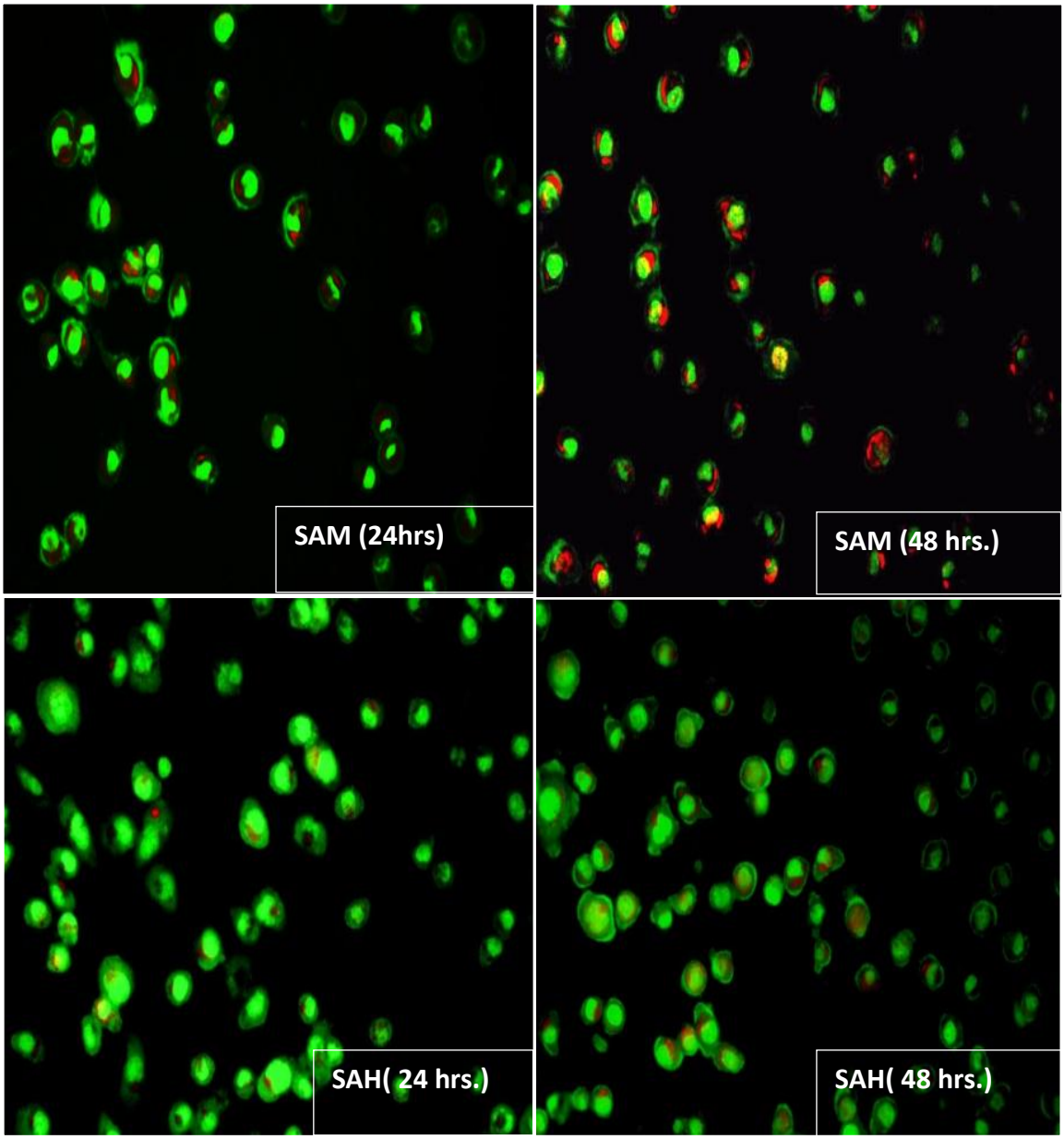

Fig 13. Fluorescence microscopic images showing formation of autophagosomes after SAM and SAH treatment

8. DNA damage measured by *Comet Assay*

MDA MB-231 cells in culture after treatment with SAM and SAH for 24-48 hr. in a time dependent manner were analyzed for the amount of DNA damage by the comet assay. The results showed significant amount of DNA damage in SAM treatment as compared to SAH and the untreated control cells. The characteristic comet tail length suggested the amount of DNA damaged. In SAM treatment the cells were seen to be having a more dispersed tail as compared to the tails of the SAH treated cells and the control untreated cells.

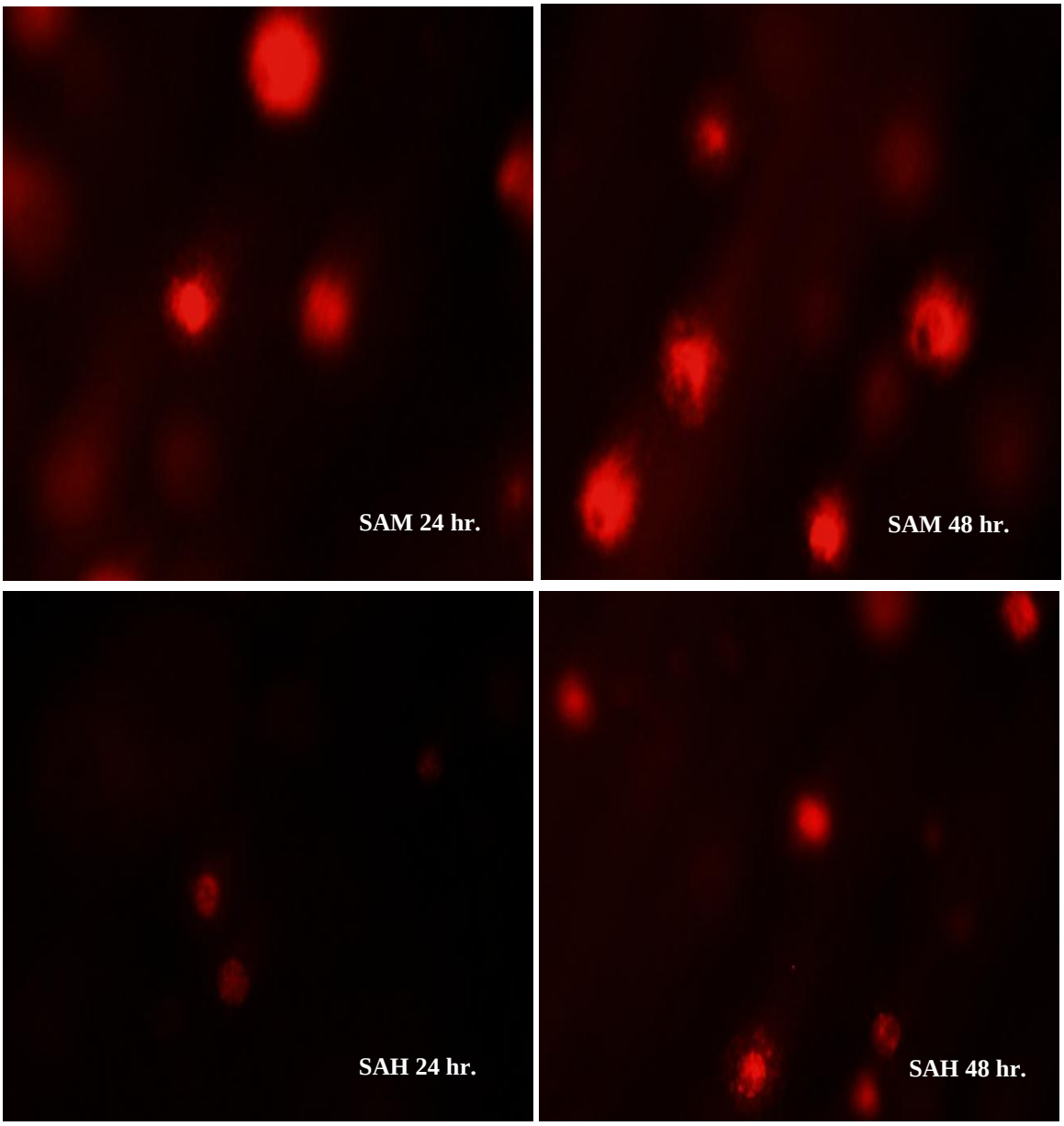

Fig 14. Fluorescence microscopic images showing DNA damage after SAM and SAH treatment

CONCLUSION

Work over the last decade has shown that Notch signaling is aberrantly activated in breast cancer and that it regulates many of the cellular properties associated with transformation. This has led to significant interest in the use of Notch pathway inhibitors for breast cancer treatment, especially as they are expected to have effects in bulk tumor cells, cancer stem cells and the surrounding tumor stroma. The question now is how best to use these inhibitors in clinical trials. Current preclinical work indicates that Notch pathway inhibitors are unlikely to be effective on their own, but that they should significantly increase the efficacy of current therapies. This said, there is still a need to identify patients that are likely to respond to Notch pathway inhibitors (He et al. 2011; Watters et al. 2009). From our study we found that after treating the MDA MB-231 metastatic breast cancer cell line with epigenetic modulators SAM and SAH , the Notch signaling components Notch1 and Hes1 are down regulated after SAM treatment and were significantly upregulated after SAH treatment. This was accompanied by changes in cellular phenotypes of the cells as the SAM treatment changed the cells to more round shape, Reduced migratory potential , Induced autophagosome formation , Aggravated chromatin condensation , Caused DNA damage as compared to SAH treated cells.

From these above experimental results we can tentatively conclude that SAM treatment of invasive breast cancer cell line induced cell death in a notch dependent manner. This experimental data suggests that SAM can be a potent therapeutic agent for treatment of breast cancer.

REFERNCES

Aktas, S., Zadeoglulari, Z., Ercetin, P., and Olgun, N. (2010). The effect of differentiating and apoptotic agents on notch signalling pathway in hepatoblastoma. *Hepatogastroenterology* **57**, 891-8.

Belyea, B. C., Naini, S., Bentley, R. C., and Linardic, C. M. (2011). Inhibition of the Notch-Hey1 axis blocks embryonal rhabdomyosarcoma tumorigenesis. *Clin Cancer Res* **17**, 7324-36.

Berman, J. N., and Look, A. T. (2007). Targeting transcription factors in acute leukemia in children. *Curr Drug Targets* **8**, 727-37.

Camps, J., Pitt, J. J., Emons, G., Hummon, A. B., Case, C. M., Grade, M., Jones, T. L., Nguyen, Q. T., Ghadimi, B. M., Beissbarth, T., Difilippantonio, M. J., Caplen, N. J., and Ried, T. (2013). Genetic Amplification of the NOTCH Modulator LNX2 Upregulates the WNT/beta-Catenin Pathway in Colorectal Cancer. *Cancer Res* **73**, 2003-13.

Chen, H. T., Cai, Q. C., Zheng, J. M., Man, X. H., Jiang, H., Song, B., Jin, G., Zhu, W., and Li, Z. S. (2012). High expression of delta-like ligand 4 predicts poor prognosis after curative resection for pancreatic cancer. *Ann Surg Oncol* **19 Suppl 3**, S464-74.

Chiron, D., Maiga, S., Descamps, G., Moreau, P., Le Gouill, S., Marionneau, S., Ouiller, T., Moreaux, J., Klein, B., Bataille, R., Amiot, M., and Pellat-Deceunynck, C. (2012). Critical role of the NOTCH ligand JAG2 in self-renewal of myeloma cells. *Blood Cells Mol Dis* **48**, 247-53.

Di Stefano, L., Walker, J. A., Burgio, G., Corona, D. F., Mulligan, P., Naar, A. M., and Dyson, N. J. (2011). Functional antagonism between histone H3K4 demethylases in vivo. *Genes Dev* **25**, 17-28.

Ferres-Marco, D., Gutierrez-Garcia, I., Vallejo, D. M., Bolivar, J., Gutierrez-Avino, F. J., and Dominguez, M. (2006). Epigenetic silencers and Notch collaborate to promote malignant tumours by Rb silencing. *Nature* **439**, 430-6.

Fraga, M. F., Berdasco, M., Ballestar, E., Ropero, S., Lopez-Nieva, P., Lopez-Serra, L., Martin-Subero, J. I., Calasanz, M. J., Lopez de Silanes, I., Setien, F., Casado, S., Fernandez, A. F., Siebert, R., Stifani, S., and Esteller, M. (2008). Epigenetic inactivation of the Groucho homologue gene TLE1 in hematologic malignancies. *Cancer Res* **68**, 4116-22.

Gasperini, P., Espigol-Frigole, G., McCormick, P. J., Salvucci, O., Maric, D., Uldrick, T. S., Polizzotto, M. N., Yarchoan, R., and Tosato, G. (2012). Kaposi sarcoma herpesvirus promotes endothelial-to-mesenchymal transition through Notch-dependent signaling. *Cancer Res* **72**, 1157-69.

Ghoshal, P., Nganga, A. J., Moran-Giuati, J., Szafranek, A., Johnson, T. R., Bigelow, A. J., Houde, C. M., Avet-Loiseau, H., Smiraglia, D. J., Ersing, N., Chanan-Khan, A. A., and Coignet, L. J. (2009). Loss of the SMRT/NCoR2 corepressor correlates with JAG2 overexpression in multiple myeloma. *Cancer Res* **69**, 4380-7.

Gorantla, B., Bhoopathi, P., Chetty, C., Gogineni, V. R., Sailaja, G. S., Gondi, C. S., and Rao, J. S. (2013). Notch signaling regulates tumor-induced angiogenesis in SPARC-overexpressed neuroblastoma. *Angiogenesis* **16**, 85-100.

Gungor, C., Zander, H., Effenberger, K. E., Vashist, Y. K., Kalinina, T., Izbicki, J. R., Yekebas, E., and Bockhorn, M. (2011). Notch signaling activated by replication stress-induced expression of midkine drives epithelial-mesenchymal transition and chemoresistance in pancreatic cancer. *Cancer Res* **71**, 5009-19.

Guo, D., Ye, J., Dai, J., Li, L., Chen, F., Ma, D., and Ji, C. (2009). Notch-1 regulates Akt signaling pathway and the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 in T-ALL cell lines. *Leuk Res* **33**, 678-85.

Hashimoto, Y., Akiyama, Y., Otsubo, T., Shimada, S., and Yuasa, Y. (2010). Involvement of epigenetically silenced microRNA-181c in gastric carcinogenesis. *Carcinogenesis* **31**, 777-84.

Henken, F. E., De-Castro Arce, J., Rosl, F., Bosch, L., Meijer, C. J., Snijders, P. J., and Steenbergen, R. D. (2012). The functional role of Notch signaling in HPV-mediated transformation is dose-dependent and linked to AP-1 alterations. *Cell Oncol (Dordr)* **35**, 77-84.

Houde, C., Li, Y., Song, L., Barton, K., Zhang, Q., Godwin, J., Nand, S., Toor, A., Alkan, S., Smadja, N. V., Avet-Loiseau, H., Lima, C. S., Miele, L., and Coignet, L. J. (2004). Overexpression of the NOTCH ligand JAG2 in malignant plasma cells from multiple myeloma patients and cell lines. *Blood* **104**, 3697-704.

Hu, W., Lu, C., Dong, H. H., Huang, J., Shen, D. Y., Stone, R. L., Nick, A. M., Shahzad, M. M., Mora, E., Jennings, N. B., Lee, S. J., Roh, J. W., Matsuo, K., Nishimura, M., Goodman, B. W., Jaffe, R. B., Langley, R. R., Deavers, M. T., Lopez-Berestein, G., Coleman, R. L., and Sood, A. K. (2011). Biological roles of the Delta family Notch ligand Dll4 in tumor and endothelial cells in ovarian cancer. *Cancer Res* **71**, 6030-9.

Hughes, D. P. (2009). How the NOTCH pathway contributes to the ability of osteosarcoma cells to metastasize. *Cancer Treat Res* **152**, 479-96.

Ivan, C., Hu, W., Bottsford-Miller, J., Zand, B., Dalton, H. J., Liu, T., Huang, J., Nick, A. M., Lopez-Berestein, G., Coleman, R. L., Baggerly, K. A., and

Sood, A. K. (2013). Epigenetic analysis of the Notch superfamily in high-grade serous ovarian cancer. *Gynecol Oncol* **128**, 506-11.

Jin, H. Y., Zhang, H. Y., Wang, X., Xu, J., and Ding, Y. (2012). Expression and clinical significance of Notch signaling genes in colorectal cancer. *Tumour Biol* **33**, 817-24.

Jithesh, P. V., Risk, J. M., Schache, A. G., Dhanda, J., Lane, B., Liloglou, T., and Shaw, R. J. (2013). The epigenetic landscape of oral squamous cell carcinoma. *Br J Cancer* **108**, 370-9.

Kang, M., Jiang, B., Xu, B., Lu, W., Guo, Q., Xie, Q., Zhang, B., Dong, X., Chen, D., and Wu, Y. (2013). Delta like ligand 4 induces impaired chemo-drug delivery and enhanced chemoresistance in pancreatic cancer. *Cancer Lett* **330**, 11-21.

Kar, S., Deb, M., Sengupta, D., Shilpi, A., Bhutia, S. K., and Patra, S. K. (2012). Intricacies of hedgehog signaling pathways: a perspective in tumorigenesis. *Exp Cell Res* **318**, 1959-72.

Kim, S. H., Sehrawat, A., and Singh, S. V. (2012). Notch2 activation by benzyl isothiocyanate impedes its inhibitory effect on breast cancer cell migration. *Breast Cancer Res Treat* **134**, 1067-79.

Koch, U., and Radtke, F. (2011). Notch in T-ALL: new players in a complex disease. *Trends Immunol* **32**, 434-42.

Koukourakis, M. I., Giatromanolaki, A., Sivridis, E., Gatter, K. C., and Harris, A. L. (2012). High DLL4 expression in tumour-associated vessels predicts for favorable radiotherapy outcome in locally advanced squamous cell head-neck cancer (HNSCC). *Angiogenesis*.

Krikelis, D., Pentheroudakis, G., Goussia, A., Siozopoulou, V., Bobos, M., Petrakis, D., Stoyianni, A., Golfinopoulos, V., Cervantes, A., Ciuleanu, T., Fountzilas, G., Malamou-Mitsi, V., and Pavlidis, N. (2012). Profiling immunohistochemical expression of NOTCH1-3, JAGGED1, cMET, and phospho-MAPK in 100 carcinomas of unknown primary. *Clin Exp Metastasis* **29**, 603-14.

Lee, J., Sehrawat, A., and Singh, S. (2012). Withaferin A causes activation of Notch2 and Notch4 in human breast cancer cells. *Breast Cancer Research and Treatment* **136**, 45-56.

Liu, M., Lee, D. F., Chen, C. T., Yen, C. J., Li, L. Y., Lee, H. J., Chang, C. J., Chang, W. C., Hsu, J. M., Kuo, H. P., Xia, W., Wei, Y., Chiu, P. C., Chou, C. K., Du, Y., Dhar, D., Karin, M., Chen, C. H., and Hung, M. C. (2012). IKKalpha activation of NOTCH links tumorigenesis via FOXA2 suppression. *Mol Cell* **45**, 171-84.

Ma, Y., Ren, Y., Han, E. Q., Li, H., Chen, D., Jacobs, J. J., Gitelis, S., O'Keefe, R. J., Konttinen, Y. T., Yin, G., and Li, T. F. (2013). Inhibition of the Wnt-beta-

catenin and Notch signaling pathways sensitizes osteosarcoma cells to chemotherapy. *Biochem Biophys Res Commun* **431**, 274-9.

Mizugaki, H., Sakakibara-Konishi, J., Ikezawa, Y., Kikuchi, J., Kikuchi, E., Oizumi, S., Dang, T. P., and Nishimura, M. (2012). gamma-Secretase inhibitor enhances antitumour effect of radiation in Notch-expressing lung cancer. *Br J Cancer* **106**, 1953-9.

Palermo, R., Checquolo, S., Giovenco, A., Grazioli, P., Kumar, V., Campese, A. F., Giorgi, A., Napolitano, M., Canettieri, G., Ferrara, G., Schinina, M. E., Maroder, M., Frati, L., Gulino, A., Vacca, A., and Screpanti, I. (2012). Acetylation controls Notch3 stability and function in T-cell leukemia. *Oncogene* **31**, 3807-17.

Patra, S. K., Patra, A., Rizzi, F., Ghosh, T. C., and Bettuzzi, S. (2008). Demethylation of (Cytosine-5-C-methyl) DNA and regulation of transcription in the epigenetic pathways of cancer development. *Cancer Metastasis Rev* **27**, 315-34.

Patra, S. K., Patra, A., Zhao, H., and Dahiya, R. (2002). DNA methyltransferase and demethylase in human prostate cancer. *Mol Carcinog* **33**, 163-71.

Pfister, S., and Witt, O. (2009). Pediatric gliomas. *Recent Results Cancer Res* **171**, 67-81.

Piazzi, G., Bazzoli, F., and Ricciardiello, L. (2012). Epigenetic silencing of Notch signaling in gastrointestinal cancers. *Cell Cycle* **11**, 4323-7.

Piazzi, G., Fini, L., Selgrad, M., Garcia, M., Daoud, Y., Wex, T., Malfertheiner, P., Gasbarrini, A., Romano, M., Meyer, R. L., Genta, R. M., Fox, J. G., Boland, C. R., Bazzoli, F., and Ricciardiello, L. (2011). Epigenetic regulation of Delta-Like1 controls Notch1 activation in gastric cancer. *Oncotarget* **2**, 1291-301.

Raimondi, L., Ciarapica, R., De Salvo, M., Verginelli, F., Gueguen, M., Martini, C., De Sio, L., Cortese, G., Locatelli, M., Dang, T. P., Carlesso, N., Miele, L., Stifani, S., Limon, I., Locatelli, F., and Rota, R. (2012). Inhibition of Notch3 signalling induces rhabdomyosarcoma cell differentiation promoting p38 phosphorylation and p21(Cip1) expression and hampers tumour cell growth in vitro and in vivo. *Cell Death Differ* **19**, 871-81.

Ranganathan, P., Weaver, K. L., and Capobianco, A. J. (2011). Notch signalling in solid tumours: a little bit of everything but not all the time. *Nat Rev Cancer* **11**, 338-51.

Ravindran, G., and Devaraj, H. (2012). Aberrant expression of beta-catenin and its association with DeltaNp63, Notch-1, and clinicopathological factors in oral squamous cell carcinoma. *Clin Oral Investig* **16**, 1275-88.

Seke Etet, P. F., Vecchio, L., and Nwabo Kamdje, A. H. (2012). Interactions between bone marrow stromal microenvironment and B-chronic

lymphocytic leukemia cells: any role for Notch, Wnt and Hh signaling pathways? *Cell Signal* **24**, 1433-43.

Stockhausen, M. T., Sjolund, J., Manetopoulos, C., and Axelson, H. (2005). Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells. *Br J Cancer* **92**, 751-759.

Subramaniam, D., Ponnurangam, S., Ramamoorthy, P., Standing, D., Battafarano, R. J., Anant, S., and Sharma, P. (2012). Curcumin induces cell death in esophageal cancer cells through modulating Notch signaling. *PLoS One* **7**, e30590.

Teider, N., Scott, D. K., Neiss, A., Weeraratne, S. D., Amani, V. M., Wang, Y., Marquez, V. E., Cho, Y. J., and Pomeroy, S. L. (2010). Neuralized1 causes apoptosis and downregulates Notch target genes in medulloblastoma. *Neuro Oncol* **12**, 1244-56.

Vartanian, A., Gatsina, G., Grigorieva, I., Solomko, E., Dombrovsky, V., Baryshnikov, A., and Stepanova, E. (2012). The involvement of Notch signaling in melanoma vasculogenic mimicry. *Clin Exp Med*.

Wang, Z., Li, Y., Ahmad, A., Azmi, A. S., Banerjee, S., Kong, D., and Sarkar, F. H. (2010). Targeting Notch signaling pathway to overcome drug resistance for cancer therapy. *Biochim Biophys Acta* **1806**, 258-67.

Wei, Y., Zhang, Z., Liao, H., Wu, L., Wu, X., Zhou, D., Xi, X., Zhu, Y., and Feng, Y. (2012). Nuclear estrogen receptor-mediated Notch signaling and GPR30-mediated PI3K/AKT signaling in the regulation of endometrial cancer cell proliferation. *Oncol Rep* **27**, 504-10.

Ye, Q. F., Zhang, Y. C., Peng, X. Q., Long, Z., Ming, Y. Z., and He, L. Y. (2012). siRNA-mediated silencing of Notch-1 enhances docetaxel induced mitotic arrest and apoptosis in prostate cancer cells. *Asian Pac J Cancer Prev* **13**, 2485-9.

Yu, B., Wei, J., Qian, X., Lei, D., Ma, Q., and Liu, Y. (2012a). Notch1 signaling pathway participates in cancer invasion by regulating MMPs in lingual squamous cell carcinoma. *Oncol Rep* **27**, 547-52.

Yu, S. D., Liu, F. Y., and Wang, Q. R. (2012b). Notch inhibitor: a promising carcinoma radiosensitizer. *Asian Pac J Cancer Prev* **13**, 5345-51.

Yustein, J. T., Liu, Y. C., Gao, P., Jie, C., Le, A., Vuica-Ross, M., Chng, W. J., Eberhart, C. G., Bergsagel, P. L., and Dang, C. V. (2010). Induction of ectopic Myc target gene JAG2 augments hypoxic growth and tumorigenesis in a human B-cell model. *Proc Natl Acad Sci U S A* **107**, 3534-9.

Zhang, J. X., Cai, M. B., Wang, X. P., Duan, L. P., Shao, Q., Tong, Z. T., Liao, D. Z., Li, Y. Y., Huang, M. Y., Zeng, Y. X., and Shao, J. Y. (2013). Elevated DLL4

expression is correlated with VEGF and predicts poor prognosis of nasopharyngeal carcinoma. *Med Oncol* **30**, 390.

Zhang, T. H., Liu, H. C., Liang, Y. J., Liang, L. Z., Zheng, G. S., Huang, H. Z., Wu, J. N., and Liao, G. Q. (2012). Suppression of tongue squamous cell carcinoma growth by inhibition of Jagged1 in vitro and in vivo. *J Oral Pathol Med*.

Zhou, L., Wang, D. S., Li, Q. J., Sun, W., Zhang, Y., and Dou, K. F. (2012). The Down-Regulation of Notch1 Inhibits the Invasion and Migration of Hepatocellular Carcinoma Cells by Inactivating the Cyclooxygenase-2/Snail/E-cadherin Pathway In Vitro. *Dig Dis Sci*.